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THE MICROBIOLOGICAL ASSAY OF TRYPTOPHANE IN PROTEINS AND FOODS

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In recent papers (1–5) methods for the assay of amino acids by bacteria have been presented. The microbiological assay of tryptophane in proteins involves special features, some of which have been described in a report (3) from this laboratory. Tryptophane must be made available to the test organism and the possibility that other substances in proteins may substitute for tryptophane must be excluded. Hydrolysis by mineral acids, which accomplishes the first objective for most amino acids, destroys tryptophane. This fact made it necessary to resort to enzymatic or alkaline hydrolysis for the preparation of test samples. *Lactobacillus arabinosus* 17-5 was found to exhibit a sensitive and reproducible response to *l*-tryptophane. In a study (3) of the specificity of this response, it was found that *d*-tryptophane, skatole, indole-3-acetic acid, β-(indole-3)-propionic acid, γ-(indole-3)-*n*-butyric acid, tryptamine hydrochloride, and kynurenic acid were inactive.[1] Indole and anthranilic acid, which were shown to be active (6, 3), could be separated from tryptophane by extraction with ether. These principles have been utilized in developing a microbiological assay procedure which appears to be reliable for the determination of tryptophane in proteins and foods.

EXPERIMENTAL

Organism—In the management of stock cultures of *Lactobacillus arabinosus* 17-5,[2] the accepted procedures of Snell and Wright (7) are followed. For the production of inoculum, the basal medium plus 0.5 mg of *l*-tryptophane for 10 cc. is used.

Basal Medium—The composition of the basal medium (Table I) for tryptophane assay differs essentially from that employed in the Snell and Wright niacin assay method (7) only in the omission of tryptophane and the inclusion of niacin. For the preparation of the hydrolyzed casein supplement, 200 gm. of casein (vitamin-free, Smaco) are hydrolyzed with 1000 cc. of 20 per cent hydrochloric acid by boiling under a reflux for 8 hours After

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[1] Kynurenine sulfate also has been found to be inactive at a level of 100 γ The authors are indebted to Dr. R. W. Jackson, Eastern Regional Research Laboratory, United States Department of Agriculture, for this substance

[2] The original culture was secured from the American Type Culture Collection, School of Medicine, Georgetown University, Washington, D. C.

distillation *in vacuo* until a thick syrup remains, 750 cc of water are added and the distillation is repeated. The residue is dissolved in water and the solution is adjusted with sodium hydroxide to pH 3 and diluted to 2000 cc. The solution is stirred with 40 gm. of norit A at 50–60° for 1 hour and filtered. The filtrate is adjusted to pH 6.8 with sodium hydroxide. The volume is readjusted to 2000 cc. and the solution is kept under toluene.

TABLE I

Composition of Tryptophane-Free Basal Medium

	per cent
Charcoal-treated hydrolyzed casein	. 0.5
Cystine	0.01
Glucose	1.0
Sodium acetate .	0.6
	mg per cent
Adenine sulfate	1.0
Guanine hydrochloride	1.0
Uracil ,	1.0
Thiamine hydrochloride	0.02
Niacin	0.02
Pyridoxine hydrochloride	0.02
p-Aminobenzoic acid	0.02
Calcium pantothenate	0.02
Riboflavin	0.02
Biotin	0.00004
Inorganic salt Solutions A and B,* 0.05 cc. each per 10 cc. medium	

* Solution A, KH_2PO_4 25 gm , K_2HPO_4 25 gm., water to make 250 cc.; Solution B, $MgSO_4 \cdot 7H_2O$ 10 gm , NaCl 0 5 gm , $FeSO_4 \cdot 7H_2O$ 0.5 gm , $MnSO_4 \cdot 4H_2O$ 0.5 gm., water to make 250 cc.

Treatment of Samples

The test materials embraced in this study include samples of several purified seed globulins,[3] other proteins of grades available at technical or commercial supply houses, and various animal or plant products from local markets. Dry materials are finely ground; fresh tissues are first dried at 100–105° sufficiently for grinding, or are homogenized directly with a Waring blendor.

Pancreatic Digestion—With dry proteins, 0.1 gm. is made up to a 0.1 per cent solution or suspension at pH 8.2 and 5 mg. of pancreatin (Pfanstiehl, 1:110) are added; with wet or protein-low samples, the above concentrations of protein and pancreatin are approximately maintained. The mixture is incubated at 37° for 24 hours. In some cases preliminary treatment of

[3] The samples of seed globulins (8) were obtained from Dr. E. L. Smith, Biological Laboratories, E. R. Squibb and Sons.

samples by (a) digestion with 1 per cent of pepsin (Pfanstiehl, 1:10,000) at pH 2 for 5 to 8 hours or (b) autoclaving a suspension of the sample in water for 0.5 hour at 15 pounds pressure improved pancreatic digestion. In any case the pancreatic digest is adjusted to pH 4 and extracted successively with two 100 cc. portions of ethyl ether followed by 30 cc. of toluene. The resulting solution is adjusted to pH 6.8 for assay. A blank tryptophane value for the pancreatin is obtained by autolysis and assay under the same conditions.

Barium Hydroxide Digestion—The method of barium hydroxide digestion reported previously (3) for the liberation of tryptophane from casein was

TABLE II

Recovery* of l-Tryptophane after Barium ˙Hydroxide Digestion

Material assayed	l-Tryptophane added	6 hrs		10 hrs	
		Found	Recovery	Found	Recovery
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Control†			97		93
Casein	0.00	1.07	93	1.02	94
	1.00	2.00		1.96	
Beef	0.00	1.15		1.09	
	1.00	2 21	106	2.13	104
Peas	0.00	0.17		0.18	
	1.00	1.24	107	1.22	104
Corn	0.00	0.066		0.069	
	1.00	1 17	110	1.09	102

* All recovery values were obtained by multiplication of direct assay values by 2 to correct for racemization   Results are expressed in terms of air-dried materials as assayed, as in Tables III and IV

† Pure l-tryptophane.

studied by recovery experiments with pure tryptophane and protein materials plus tryptophane. It was found that recoveries of tryptophane were approximately constant when the samples were autoclaved with 5 N barium hydroxide for 6 to 10 or even 15 hours. When multiplied by 2 to correct for racemization, the results (Table II) indicated quantitative recovery of tryptophane. Results obtained after 4 hours digestion with barium hydroxide indicated incomplete racemization of tryptophane. On the basis of these experiments the following procedure was adopted for the preparation of samples: 0.5 gm. of powdered sample is mixed with 4.2 gm. of anhydrous barium hydroxide and 8 cc. of water in a 25 cc. Pyrex Erlenmeyer flask and autoclaved for 7 hours at 15 pounds pressure. The hydrolysate is transferred with the aid of hot water and a rubber policeman to a 250 cc. centrifuge bottle, and 10 N sulfuric acid is added until pH 4 is reached. The

mixture is then diluted to 101.5 cc. (the barium sulfate occupies a volume of 1.5 cc.), mixed thoroughly at 50–60°, covered, and centrifuged. The separated liquid is cooled and extracted successively with two 100 cc. portions of ethyl ether followed by 30 cc. of toluene. The resulting solution is adjusted to pH 6.8 for assay.

Assay Procedure

The response of *Lactobacillus arabinosus* to *l*-tryptophane (Fig. 1) shows a rise in production of 0.1 N acid from a negative control of about 0.3 cc. to over 9 cc. in the presence of 20 γ of tryptophane. In obtaining a standard curve for assay, *l*-tryptophane (Merck) is used at levels of 0, 2, 4, 6, 8, 10, and 12 γ per assay tube. Dilutions are prepared as desired from a stock
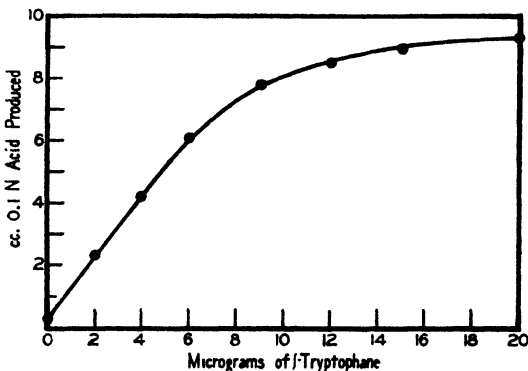
FIG. 1. Response of *Lactobacillus arabinosus* to *l*-tryptophane

solution containing 1 mg. per cc., which is kept cold under toluene and renewed at 2 week intervals.

It has been the general practice to prepare the basal medium at twice the concentrations shown in Table I, allowing for additions of standard and sample solutions before dilution of the contents of each tube to 10 cc. for assay. In the assay of materials fairly rich in tryptophane a more convenient procedure may be followed. The basal medium is made directly to full volume and is distributed into test-tubes in 10 cc. portions. Suitable amounts of standard and sample solutions, not exceeding 0.6 cc. per tube, are then added. The autoclaving, inoculation, incubation at 37° for 72 hours, and measurement of response by titration follow recognized procedures (7).

Assay Results

When pancreatin is used for the preparation of samples, direct assay results require correction for the tryptophane content of the pancreatin,

while results based on barium hydroxide treatment are multiplied by 2 to correct for racemization. The data presented (Tables II to IV) as evidence of the reliability of this assay method are based on the use of barium hydroxide, since it appears to be more satisfactory for the preparation of materials for assay,

The results in Table II have been cited as evidence of the quantitative recovery of tryptophane added to natural products. When assay results

TABLE III

Tryptophane Content of Materials Calculated from Different Assay Levels
Direct assay results after barium hydroxide digestion were multiplied by 2.

Lima beans			Linseed meal			Lamb muscle		
Amount per assay tube	Tryptophane		Amount per assay tube	Tryptophane		Amount per assay tube	Tryptophane	
	Found	Content		Found	Content		Found	Content
mg.	γ	*per cent*	*mg*	γ	*per cent*	*mg.*	γ	*per cent*
1.8	2.2	0.24	0.9	2.95	0.65	0.5	2.9	1.16
3.6	4.1	0.23	1.8	5.5	0.61	1.0	5.8	1.16
5.4	6.8	0.25	2.7	8.1	0.60	1.5	7.85	1.05
Average.		0.24			0.62			1.12

TABLE IV

Reproducibility of Assays
Direct assay results after barium hydroxide digestion were multiplied by 2.

Material	Tryptophane content			
	Assay 1	Assay 2	Assay 3	Average
	per cent	*per cent*	*per cent*	*per cent*
Soy bean protein	1.02	0.96	0.98	0.99
Gliadin..	0.62	0.56	0.58	0.59
Whole milk powder......	0.32	0.30	0.31	0.31
Lactalbumin	1.57	1.62	1.59	1.59
Rolled oats..	0.20	0.21	0.20	0.20

are calculated from different sample levels (Table III), there is excellent agreement and no significant drift with increase in the size of the sample. In repetition (Table IV) of assays of a given sample the replicability of results is satisfactory. Table V contains microbiological assay results for tryptophane and also nitrogen contents, calculated on a moisture-free basis, of a number of proteins and foods of various types. Assay results based on barium hydroxide digestion are compared in many cases with data based on pancreatic digestion.

TABLE V

Microbiological Values for Tryptophane in Proteins and Foods

The samples were digested with pancreatin or barium hydroxide for tryptophane assay.

Material	Nitro-gen*	Tryptophane,* actual		Tryptophane, based on 16.0 per cent N			Bibliographic reference No.
		Pan-creatin	Bar-ium hy-drox-ide	Pan-creatin	Bar-ium hy-dro-xide	Literature†	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Casein A . .	14.55	1.06	1.09	1.17	1.20	⎫	
" B . .	14.18	1.07	1.10	1.21	1.24	⎬	
" C	14.76	1.10	1.09	1.19	1.18	⎬1.21, 1.22, 1.3	9, 10, 11
" D .	14.78	1.09	1.11	1.18	1.20	⎬	
" E .	15.07		1.13		1.20	⎭	
Soy bean Protein A	16.16	1.05	1.05	1.04	1.04	⎫	
" " " B .	15.68		1.17		1.19	⎬1.5	12
" " " C	15.35		1.09		1.14	⎭	
Lactalbumin	13 14		1.69		2.06	2.35, 2.14	11, 13
Ovalbumin .	12.84	1.17‡	1.18	1.46	1.47	1.35, 1.26, 1.6	14, 15, 11
Seed globulin, Squash A	18.40	1.68	1.74	1.46	1.51	1.46	8
Seed globulin, Squash B	18.29	1.65	1.78	1.44	1.56		
Seed globulin, cucum-ber	17.87	1 67	1.94	1.50	1.74	1 49	8
Seed globulin, water-melon	18.36	1.79	1.87	1.56	1.63	1 54	8
Seed globulin, tobac-co	19.09	1 30	1.49	1.09	1.25	1.21	8
Gliadin	16.74	0.51	0.62	0.49	0.59		
Zein A.	14.90	0 03§	0.09	0.03	0.10		
" B .	15.08		0.08		0.09		
Gelatin	17 56		0.003				
Peptone (Bacto)	15.79	0.22	0.30	0.22	0.30		
Beef round	13 41	0.82‖	1.18	0.99	1.41	1 35	16
Pork loin	12.60	0.70‖	1.08	0.89	1.37	1.31	16
Lamb "	12.48	0 71‖	1.11	0.91	1.42	1 44	16
Beef pancreas	11 12	0 34‖	0 53	0 49	0.76	1.3	11
Dried liver	10.12	0.61	1 02	0 96	1.62	1.55	16
Whole wheat	2.91	0 09	0.21	0 50	1.16	0.41–0.55, 0.84	17, 12
White flour	1.82		0.14		1.23	0.84	12
Yellow corn	1.42	0.06	0.074	0.68	0.84	0.7	12
Rolled oats	2 45	0.15	0.22	0.98	1.44		
Polished rice	1 28		0.09		1.13	1.04	18
Peas	3.62		0.18		0.80		

TABLE V—*Concluded*

Material	Nitro-gen*	Tryptophane,* actual		Tryptophane, based on 16.0 per cent N §			Bibliographic reference No.
		Pan-creatin	Bar-ium hy-drox-ide	Pan-creatin	Bar-ium hy-drox-ide	Literature†	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Navy beans	3.63		0.24		1.06		
Lima " .	3.66		0.26		1.14		
Soy flour, fat-free	9.43	0.49	0.70	0.83	1.19	1.5	12
" " low-fat .	8.59	0.41	0.66	0.76	1.23		
Dried milk	3.91		0.32		1.31	1.32, 1.6	16, 12
" yeast .	7.40	0.38	0.61	0.82	1.32	1.4	12
Linseed meal .	6.46		0.64		1.59		

* Nitrogen and tryptophane are calculated on a moisture-free basis. Moisture was determined by drying to constant weight at 110°.

† Tryptophane values on the basis of 16.0 per cent nitrogen are cited directly from the literature or recalculated from the reported nitrogen content.

‡ Pancreatic digestion was preceded by autoclaving for 0.5 hour.

§ The sample was digested with 2 per cent of pancreatin.

‖ The sample was autoclaved for 0.5 hour, digested with pepsin for 8 hours, and then with pancreatin.

DISCUSSION

In the search for a reliable method for making tryptophane available to *Lactobacillus arabinosus*, a study of enzymes, especially pancreatin, was made. However, it appeared that the conditions for complete liberation of tryptophane by enzymes varied with the substrate, as noted by Mitchell and Hamilton (19). Furthermore, the pancreatin preparations contained 1 to 1.5 per cent of tryptophane, which introduced a considerable correction factor. On the other hand, barium hydroxide produced complete hydrolysis without significant destruction. Complete racemization took place under the experimental conditions, so that the observed values were multiplied by 2. The data in Table V show the superiority of the barium hydroxide hydrolysis. In general, the results agree with those reported in the more recent literature, when compared on a common basis. For purposes of comparison, there are included in Table V the results of this study and values from the literature, which have been calculated on the basis of a nitrogen content of 16 per cent. In some cases in which such values were not reported, it was necessary to calculate the figures from reported nitrogen contents.

The authors are indebted to Miss Lois Herbert and Miss Rachel Mato for technical assistance, to Miss Edna Heacock for supervision of stock cultures, and to Mr. E. D. Perry for nitrogen determinations. The authors also wish to thank Dr. B. F. Chow and Dr. E. L. Smith for their assistance.

SUMMARY

Previous studies of the tryptophane requirement of *Lactobacillus arabinosus* 17-5 are extended and applied to the microbiological determination of tryptophane in various materials. A comparison of barium hydroxide and pancreatin for the hydrolysis of proteins indicates that the former is more generally applicable to the preparation of samples. The method satisfies customary criteria of reliability with respect to recovery of tryptophane and concordance of results calculated from different sample levels and in independent assays. The results are in general agreement with many values obtained by chemical methods which are found in the literature.

BIBLIOGRAPHY

1. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **150**, 477 (1943); **151**, 511 (1943).
2. Kuiken, K. A., Norman, W. H., Lyman C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
3. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 322 (1943).
4. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
5. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
6. Snell, E. E , *Arch. Biochem.*, **2**, 389 (1943).
7. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
8. Vickery, H. B., Smith, E. L., Hubbell, R. B., and Nolan, L. S., *J. Biol. Chem.*, **140**, 613 (1941).
9. Toennies, G., *J. Biol. Chem.*, **145**, 667 (1942).
10. Beach, E. F., Bernstein, S. S., Hoffman, O. D., Teague, D. M., and Macy, I. G., *J. Biol. Chem.*, **139**, 57 (1941).
11. Block, R. J., and Bolling, D., The determination of the amino acids, Minneapolis, revised edition (1942).
12. Block, R. J., and Bolling, D., *Arch. Biochem.*, **3**, 217 (1943).
13. Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **31**, 1751 (1937).
14. Calvery, H. O., *J. Biol. Chem.*, **94**, 613 (1931–32).
15. Brand, E., and Kassell, B., *J. Biol. Chem.*, **131**, 489 (1939).
16. Beach, E. F., Munks, B., and Robinson, A., *J. Biol. Chem.*, **148**, 431 (1943).
17. Csonka, F. A., *J. Biol. Chem.*, **118**, 147 (1937).
18. Kik, M. C., *Cereal Chem.*, **18**, 349 (1941).
19. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, American Chemical Society monograph series, New York, 211 (1929).

A REPORTED GROWTH STIMULANT FOR
LACTOBACILLUS CASEI

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Pollack and Lindner (1) have reported the existence in natural extracts, particularly in Wilson's peptone, of a growth stimulant of unknown nature which is effective for *Lactobacillus casei*.

In the course of following up this work it was found possible to improve the basal medium used, by the addition of a mixture of known compounds. When, for example, a modified basal medium containing 1.2 mg. of pyridoxine, 0.4 mg. of *p*-aminobenzoic acid, and 0.2 gm. of asparagine per liter was used, the addition of Wilson's peptone was found not to have a stimulating effect.

The three additions mentioned were essential for a maximum response; the addition of *p*-aminobenzoic acid alone had an inhibitory effect. The increased concentration of pyridoxine was beneficial because "pseudopyridoxine" is the substance utilized by *Lactobacillus casei* (2), and it is not formed in optimal amounts unless the pyridoxine concentration in the medium is relatively high. The amount of asparagine used was relatively high and its effect when used alone was sufficiently small to justify the negative result reported by Pollack and Lindner.

Subsequent to these preliminary experiments an attempt was made to prepare a mixture containing glutamine (which was found by Pollack and Lindner to be 5 to 10 times as potent as Wilson's peptone), *p*-aminobenzoic acid, and "pyridoxal" (3) which would, when added to Pollack and Lindner's basal medium in low concentrations, simulate or reproduce the effect of adding Wilson's peptone. The extent to which this was successful is indicated in Table I. The mixture contained 25.6 parts of glutamine to 0.001 part of *p*-aminobenzoic acid, and 0.02 part of pyridoxal, and the tests were carried out in accordance with the procedure of Pollack and Lindner except that the test media were steamed instead of autoclaved before inoculation.

Separate tests not reported in detail showed that the mixture mentioned above was from 40 to 102 times as potent on a weight basis as Wilson's peptone. Since the growth-stimulating effect of the mixture is substan-

tially equivalent to that of the peptone, regardless of the manner of testing, it appears that the magnitude of the effect of the peptone might be explained if it contained from 1 to 2 per cent glutamine and small amounts of p-aminobenzoic acid and pyridoxal. However, Pollack and Lindner have indicated that glutamine is more readily hydrolyzed than the "peptone factor." This would seem to rule out glutamine as an active agent unless the possibility of its being protected from hydrolysis by combination or admixture with peptone constituents were considered seriously. The

TABLE I

Comparative Effects of Peptone and a Known Mixture

Amount of peptone in test	Amount of mixture in test	Turbidity (galvanometer divisions)
γ	γ	
0	0	46.5
100	0	63
250	0	65
375	0	68 5
500	0	66
0	0	46.5
0	2.56	56
0	17.9	59.5
0	25.6	62
0	51.2	67
500	0	66
500	2.56	64
500	5 12	75.5
500	7.7	72.5
500	12.8	75.5
0	25.6	62
125	25.6	70
250	25.6	72
375	25.6	75

fact that asparagine in sufficient quantities can take the place of glutamine, and the observation of Pollack and Lindner that a mixture of amino acids was alone stimulative, suggest that the physiological activity of the peptone is probably due to its content of p-aminobenzoic acid, material with vitamin B_6 activity, and various amino acids and peptides rather than to the presence of a single unknown "peptone factor" as postulated as a result of the previous investigation. Because of this probability and the difficulty of establishing a satisfactory test for any postulated unknown substance, attempts at isolation will not be carried forward.

SUMMARY

Evidence is presented which leads to the conclusion that the effect of Wilson's peptone in stimulating the growth of *Lactobacillus casei* observed

by Pollack and Lindner is probably due to its content of p-aminobenzoic acid, material with vitamin B_6 activity, and various amino acids and peptides rather than to the presence of a single "peptone factor."

BIBLIOGRAPHY

1. Pollack, M. A , and Lindner, M., *J. Biol. Chem.*, **147**, 183 (1943).
2. Snell, E. E., *Proc. Soc. Exp. Biol and Med.*, **51**, 356 (1943).
3. Snell, E. E., *J Biol. Chem* , **154**, 313 (1944)

THE DETERMINATION OF CHOLINE IN PHOSPHOLIPIDS

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The use of reineckate as a precipitating agent in the determination of choline was introduced by Kapfhammer and Bischoff (1). This precipitation was later adapted by Beattie to the colorimetric determination of micro quantities of choline (2). Beattie used a saturated solution of ammonium reineckate in water for the precipitation of choline and stated that the precipitation of choline at temperatures as high as 60° is complete in 10 minutes. In order to increase the concentration of reineckate in the precipitating medium, Jacobi *et al.* (3) modified Beattie's precipitation procedure by using a 2 per cent solution of ammonium reineckate in methanol; at the same time the mixture is allowed to stand in the cold for 12 hours to insure complete precipitation.

In connection with a method for the separation of choline-containing from non-choline-containing phospholipids, to be reported in the following paper, a modification was developed for measuring choline accurately and rapidly. It is shown here that the increased solubility of ammonium reineckate in 1.2 N hydrochloric acid makes possible the use of more concentrated solutions of reineckate and still permits complete precipitation of choline within 30 minutes at room temperature.

Procedure

Extraction of Tissues—Tissues are usually extracted with alcohol and ether, the alcohol-ether extracts concentrated to a low volume under reduced pressure in an atmosphere of CO_2, and the lipids redissolved in petroleum ether.[1] Portions of the petroleum ether extract are transferred to centrifuge tubes and concentrated to a low volume, and the phospholipids quantitatively precipitated with acetone and $MgCl_2$. After centrifugation the precipitated phospholipids are redissolved in a mixture of methanol and ether and transferred to a specially designed flask[2] used for hydrolysis of phospholipids.

[1] It has been shown here that petroleum ether redissolves all of the phospholipids in the alcohol-ether concentrate obtained from liver, plasma, and kidney. The aqueous phase that remained after the extraction with petroleum ether was concentrated to dryness under reduced pressure in an atmosphere of CO_2; a chloroform extract prepared from the residue contained only traces of phosphorus.

[2] The vessel consists of an Erlenmeyer flask (125 cc.) with a side arm of approximately 2 cc. capacity. The side arm is made from 10 mm. glass tubing and is sealed

Hydrolysis of Phospholipid—The methanol-ether solution of phospholipid is evaporated to about 5 cc. 15 cc. of a saturated Ba(OH)$_2$ solution are then added and the flask placed on a steam bath in direct contact with steam for about 2 hours and frequently shaken. When the mixture is brought almost to dryness (1 to 3 drops), it is acidified with 1.7 cc. of 6 N HCl and the mixture heated on the steam bath for a few moments with shaking to insure complete acidification. 15 cc. of petroleum ether are added to the flask. It is then heated on the steam bath and shaken well, and just as all bubbling ceases, the petroleum ether phase is poured off, while the aqueous phase is retained in the side arm. The aqueous phase is then extracted twice more in a similar manner with two separate 15 cc. portions of petroleum ether. The petroleum ether serves to extract the fatty acids and is discarded.

Precipitation of Choline in Centrifuge Tube—5 to 8 drops of distilled water are added to each flask and the flask warmed on the steam bath until all salts are dissolved. The warmed solution is transferred without loss to a 15 cc. graduated Pyrex centrifuge tube. The flask is washed with two 2 cc. portions of 1.2 N HCl and the washings transferred to the centrifuge tube. The final volume in the centrifuge tube amounts to 7 to 9 cc.

The reineckate solution for the precipitation of choline is prepared just before use by dissolving 2 gm. of finely powdered ammonium reineckate (Hoffmann-La Roche) in 100 cc. of 1.2 N HCl and filtering it. To the aqueous phase in the centrifuge tube are added slowly and with stirring 5 cc. of the reineckate solution and the mixture is allowed to stand for 30 minutes, during which time it is frequently agitated with a glass rod. This mixture is then centrifuged for 10 minutes at a speed of at least 2500 R.P.M. and the supernatant containing the excess reineckate is carefully decanted. While the tube is still inverted, its mouth is wiped clean in order to remove the excess reineckate as completely as possible. The stirring rod used previously is returned to the tube and approximately 3 cc. of 1.2 N HCl delivered into the tube in such a manner as to wash down its sides and the stirring rod. The mixture is stirred with the minimum of agitation required for the adequate washing of the precipitate. Prolonged agitation should be avoided, especially with small samples of choline, in order to prevent solution of the precipitate. The stirring rod is now removed and the contents of the tube centrifuged again for 10 minutes at 2500 R.P.M. The superna-

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into the flask at an angle of 45° about 3 cm. from the top. This type of flask is particularly useful for the extraction of lipids from an aqueous phase of small volume. After the aqueous phase is shaken with petroleum ether, it is allowed to stand until the petroleum ether phase is clear. The petroleum ether is then decanted, the aqueous phase being caught in the side arm. The aqueous phase is then reextracted in a similar manner several times with fresh portions of petroleum ether

tant is discarded and the mouth of the tube again wiped clean. After the choline reineckate is completely dissolved in 6 to 10 cc. of acetone, the tube is centrifuged for 5 minutes.

The volume in the centrifuge tube is read to 0.05 cc. and the solution decanted into a colorimeter tube. The latter is stoppered to prevent evapo-
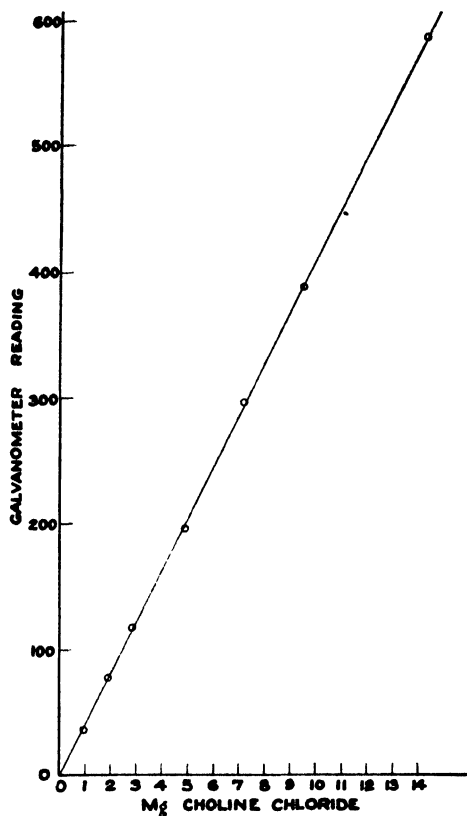
FIG 1 Plot of galvanometer readings against choline chloride

ration of the acetone. The color is determined in a Klett-Summerson photoelectric colorimeter containing the No. 54 filter (500 to 570 mμ).

Samples that contained small amounts of choline were made up to small volumes in order to obtain colors that were intense enough to be read accurately on the colorimeter and that still obeyed Beer's law. The minimum volume that could be used for a colorimetric reading is slightly over 5 cc.

Standards—In all choline determinations standard samples containing known amounts of choline were precipitated with reineckate and run along

with the unknown samples.   The standard choline solutions were prepared from choline chloride that had been recrystallized from absolute alcohol and dried in the vacuum desiccator.   An aliquot of a standard solution prepared from such choline is transferred to a 15 cc. graduated centrifuge tube, 1.4 cc. of 6 N HCl added, and the volume in the tube made up to 7 cc. with water.   The choline is precipitated as described above.   In Fig. 1 the galvanometer readings (corrected to a 10 cc. basis) have been plotted against the amounts of choline chloride precipitated.

The degree of variation in the colorimetric readings of acetone solutions of choline reineckate obtained after precipitation of standard solutions of choline chloride as described above is shown in Table I   The maximum difference between analyses carried out on eight different days amounted to about 5 per cent.   Consequently the amount of choline in an unknown solution can be determined to within 5 per cent merely by reference to the

TABLE I

Extent of Variation in Colorimeter Readings from Day to Day

Total No of analyses (8 separate experiments* each)	Choline chloride pptd	Mean colorimeter reading	Standard deviation
	mg		
21	1.90	76.5	±1.15
21	2.85	116.5	±1.87
16	4.75	196	±2.66

* Each experiment was conducted on a different day.

standard curve shown in Fig. 1   In this laboratory, however, a new standard curve was prepared with each set of choline determinations; in this way the error of each determination is probably reduced to not more than 3 per cent.

Calculations—In the calculations all the readings were converted to a basis of 10 cc. volume.   For example, a sample which at a volume of 6.4 cc. gave a colorimeter reading of 147 would, if it had been made up to 10 cc., give a reading of $6.4/10 \times 147 = 94$.   The choline values corresponding to the colorimetric readings corrected for a volume of 10 cc. were then obtained from a chart prepared after the manner described for Fig. 1.

Test of Method

Beer's Law—Standard samples containing from 0.95 to 14.25 mg. of choline chloride were precipitated in centrifuge tubes as described above and the color obtained measured in the Klett-Summerson photoelectric colorimeter.   In Fig. 1 the galvanometer readings (converted to a 10 cc. basis) have been plotted against the amounts of choline chloride precipitated.

Since the galvanometer scale of this colorimeter is graduated logarithmically, Fig. 1 shows that the colors produced over a range of 0.95 to 14.25 mg. of choline chloride obey Beer's law. *The fact that the straight line passes through the origin* may be taken as evidence that for choline samples of 1 mg. or more a significant quantity of the choline reineckate precipitate is not lost by washing the precipitate with 3 cc. of 1.2 N HCl.

TABLE II

Recovery of Choline Added to Liver Phospholipid

All choline values are expressed as the chloride.

Experiment No	Choline added to sample	Choline initially present in sample	Total choline found in sample	Recovery of the added choline	
	mg	*mg*	*mg*	*mg*	*per cent*
1	1.87	0	1.87	1.87	100
2	2.81	0	2.85	2.85	101
3	4.68	0	4.77	4.77	102
4	1.87	3.17	4 96	1.79	96
5	1.87	2 22	4 23	2.01	107
6	1.87	1.59	3 52	1.93	103
7	0.935	1.27	2 22	0.95	102
8	0.935	1 59	2.55	0 96	103
9	2.81	1.59	4.47	2.88	102
10	2.81	2 22	5.10	2.88	102

TABLE III

Effect of Duration of Hydrolysis on Amount of Choline Liberated from Liver Phospholipid

All choline values are expressed as the chloride

Sample No	Duration of hydrolysis	Choline found	Sample No	Duration of hydrolysis	Choline found
	hrs	*mg*		*hrs.*	*mg.*
1	2	2.85	2	2	4.55
	3	2.85		3	4.50
	4	2.85		4	4.55
	5	2.85		5	4 55

Barium Hydroxide Hydrolysis—In order to extract completely the hydrolyzed fats and to keep the volume of the aqueous phase small, the mixture was concentrated almost to dryness during the hydrolysis with Ba(OH)₂. It was therefore desirable to know whether this treatment affected the recovery of choline. The following experiments were carried out to test this point.

Known amounts of choline were added to 15 cc. portions of saturated Ba(OH)₂ and the mixtures treated as outlined in the "Procedure" above.

From the first three results recorded in Table II it can be seen that treatment with $Ba(OH)_2$ has no destructive action on choline.

In Experiments 4 to 10 (Table II) the choline content of a methanol-ether solution of phospholipids was determined. To another aliquot of the same solution, known amounts of a standard choline solution were then added and the total choline content measured. The results obtained indicate that added choline can be quantitatively recovered when treated with $Ba(OH)_2$ in the presence of phospholipids.

The effect of duration of hydrolysis with $Ba(OH)_2$ on the recovery of choline from phospholipids is shown in Table III. It is evident that hydrolysis is just as complete in 2 hours as it is in 5 hours. This may be taken as evidence that practically all the choline combined in liver phospholipids is liberated by the barium hydrolysis procedure used here. Further evidence for this view is supplied in the following paper, in which choline to phosphorus ratios of almost unity were obtained from phospholipid samples by barium hydroxide hydrolysis. It is unlikely that such ratios would have been obtained if the hydrolysis with the barium hydroxide were incomplete.

DISCUSSION

The present method has been limited to the measurement of the choline content of phospholipids, and no attempt has been made to apply it to other biological materials. Presumably it is subject to the same limitations as all methods in which reineckate is used as a precipitation agent for choline. It has the advantage, however, of giving complete precipitation of choline within 30 minutes at room temperature; moreover, the high concentration of reineckate in the dilute hydrochloric acid makes possible the determination of choline over a wide range

That the precipitation of choline is quantitative between 1 and 14 mg. is suggested by the observation that the straight line shown in Fig. 1 passes through the origin. Triplicate values that agree within 3 per cent were obtained from analyses of samples containing 1 to 14 mg. of choline chloride. When the samples analyzed contained less than 1 mg. of choline chloride, there was a tendency for the recoveries to be low, an observation that indicates that some loss occurs when small amounts of precipitated choline are washed with hydrochloric acid.

SUMMARY

A rapid and accurate method for measuring choline by precipitation with reineckate in dilute hydrochloric acid is presented.

BIBLIOGRAPHY

1. Kapfhammer, J., and Bischoff, C., Z. physiol. Chem., **191**, 179 (1930).
2. Beattie, F. J. R., Biochem. J., **30**, 1154 (1936).
3. Jacobi, H. P., Baumann, C. A., and Meek, W. J., J. Biol. Chem., **138**, 571 (1941)

AN ADSORPTION PROCEDURE FOR THE SEPARATION OF CHOLINE-CONTAINING FROM NON-CHOLINE-CONTAINING PHOSPHOLIPIDS OF LIVER

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Investigations in phospholipid metabolism have been handicapped by lack of a satisfactory method for the resolution of mixtures of tissue phospholipids. Various methods for separating phospholipids have been suggested, but none has proved to be entirely satisfactory.

Petroleum ether was used for separating lecithin and cephalin from sphingomyelin by Hevesy and Hahn (1) and by Chargaff (2). These workers claimed that, when a lipid extract was concentrated to dryness and the residue treated with cold petroleum ether, the lecithins and cephalins were dissolved and thereby separated from the insoluble sphingomyelin. The cephalin fraction was then separated from the lecithin by precipitation with alcohol. The individual phospholipid fractions were further purified by reprecipitation. Since these investigators (using radioactive P) were primarily interested in the specific activities of the various phospholipid fractions, they were not concerned with the loss of material involved in the purification of the individual fractions. Unfortunately, no data were presented on the effectiveness of the separation, and so it is difficult to evaluate this procedure. The use of alcohol for the precipitation of cephalin, however, is contraindicated by the finding of Folch·(3) that phosphatidyl ethanolamine is freely soluble in alcohol. Moreover, the methods used by Hevesy and Hahn and by Chargaff are not satisfactory when only small amounts of phospholipid are available or when information on the actual amounts of the different phospholipids is desired.

Another method of separation was introduced by Kirk (4). In this method the phospholipid mixture is first precipitated with acetone and $MgCl_2$ and then extracted with moist ethyl ether. According to Kirk, moist ethyl ether dissolves lecithin and cephalin but not sphingomyelin. This method has been criticized by Sinclair and Dolan (5) and by Ramsay and Stewart (6). Sinclair and Dolan maintain that the phospholipid fraction not dissolved by treatment of the precipitated phospholipids with moist ethyl ether does not consist primarily of sphingomyelin but is merely a portion of the mixture of all phospholipids present. Furthermore, they report that the percentage of ether-insoluble phospholipids is a function of the amount of $MgCl_2$ added during the precipitation with acetone.

Reinecke salt as a specific precipitating agent for sphingomyelin was first used by Thannhauser and Setz (7) and subsequently adopted by a number of other workers (8–13). Erickson *et al.* (9) and Hunter (11) have presented data in support of this method. Employing samples of sphingomyelin that were 70 to 90 per cent pure, the former reported recoveries of 76 to 113 per cent by this procedure. When known amounts of the sphingomyelin preparation were added to stroma extracts, the recoveries varied between 97 and 110 per cent. These results do not preclude the possibility of precipitation of phospholipids other than sphingomyelin when reineckate is added to a mixture of phospholipids. As yet, there is no conclusive evidence that reineckate precipitates sphingomyelin quantitatively and specifically (*i.e.* free from other phospholipids) from lipid extracts of tissue. In rats maintained on a uniform diet Artom and Fishman (13) found such widely varying amounts as 10 and 680 mg. per cent of liver sphingomyelin by the reineckate precipitation method. Indeed, they state that more work is required to establish the significance of their data on sphingomyelin.

The determination of the individual phospholipids in mixtures need not involve the actual separation of any one of them. Ramsay and Stewart (6), for example, have presented data on the distribution of the separate phospholipids in human whole blood based on measurements of lipoid P, choline, and glycerol. They obtained values for sphingomyelin considerably higher than other investigators. Blix (14) determined lipoid P and glycerol in samples of human serum and calculated the non-glycerol phospholipid (sphingomyelin) content, which he found to be about 13 per cent of the total phospholipid.

In the present communication a new approach to the separation of phospholipids is presented. Preliminary work[1] suggested that phospholipids in petroleum ether solution could be adsorbed on magnesium oxide and that lecithin and sphingomyelin could be eluted with methyl alcohol. This adsorption phenomenon was then studied in detail and incorporated into a procedure for the separation of choline-containing (lecithin and sphingomyelin) from non-choline-containing phospholipids (cephalins) of liver.

<div style="text-align: center;">EXPERIMENTAL</div>

Extraction of Phospholipids from Liver—The extraction of phospholipids from liver was carried out as follows: The weighed tissue was ground with sand in a mortar, transferred to a flask, and extracted with alcohol at 55–60° for 2 hours with occasional shaking. Approximately 150 cc. of alcohol were used for 20 to 25 gm. of liver. The supernatant alcohol was decanted through filter paper and the residue extracted with a second portion of alcohol for 1 hour. The contents of the flask were then poured through the

[1] Fries, B. A., and Chaikoff, I. L., unpublished observations.

same filter paper and the two alcohol extracts combined. The tissue residue was then extracted overnight with ether in a Soxhlet apparatus and the ether extract added to the alcohol extracts. The combined alcohol-ether extracts were concentrated to a small volume (3 to 4 cc.). This was carried out in a hot water bath (55–60°) under reduced pressure and in an atmosphere of CO_2. This concentrate was extracted with several portions of petroleum ether (b.p. 30–60°) and the petroleum ether extract made up to a desired volume by the addition of more petroleum ether.

Adsorption of Phospholipid on Magnesium Oxide from Petroleum Ether Solution—The amounts of phospholipid adsorbed from a petroleum ether solution by various amounts of magnesium oxide are shown in Table I. Merck's U. S. P. light magnesium oxide was used throughout. Equal volumes of a petroleum ether extract of liver lipids ·were added to amounts of MgO varying from 0.3 to 1.0 gm. in 50 cc. centrifuge tubes. The MgO-

TABLE I

Adsorption of Dog Liver Phospholipids from Petroleum Ether Solution by Magnesium Oxide

To the amounts of MgO recorded below were added 15 cc of a petroleum ether solution containing 0.85 mg. of phospholipid P.

MgO used for adsorption, *gm*	0 3	0 4	0 5	0 6	0.75	1.0
Per cent phospholipid P adsorbed	82	96	100	100	100	100

petroleum ether mixtures were stirred frequently for 15 to 20 minutes and then centrifuged. The supernatant petroleum ether was poured off and evaporated to dryness, and the phosphorus determined by King's method (15). The results shown in Table I indicate that all the phospholipid contained in 15 cc. of the petroleum ether solution (approximately 21 mg. of phospholipid) was adsorbed on 0.5 gm. of MgO. When the quantity of the phospholipid solution and the amount of MgO were proportionally increased, the adsorption was still complete. The concentration of the phospholipid in the petroleum ether was kept nearly constant in all experiments, about 0.06 mg. of phospholipid P per cc.

Elution of Phospholipids—The experiments of Table II were carried out as follows: 25 cc. of petroleum ether solutions containing approximately 0.060 mg. of phosphorus per cc. were added to 1.0 or 1.75 gm. of MgO in a 50 cc. centrifuge tube. The mixture was stirred at frequent intervals for 15 to 20 minutes and then centrifuged. The supernatant, which contained no phosphorus, was discarded. The residue was washed with 25 cc. of fresh petroleum ether and the mixture centrifuged again. The supernatant fluid was again discarded.

The MgO was now subjected to several treatments with methyl alcohol.

In the first treatment 30 cc. of the alcohol were added and the mixture agitated at frequent intervals for 20 minutes. The mixture was then centrifuged and the supernatant decanted. The residue was washed twice more with 25 cc. portions of methyl alcohol and the mixture stirred repeatedly for 20 minutes after each addition. The methyl alcohol eluates were then combined and made up to volume. Aliquots of the latter were taken for determinations of phosphorus and choline. Choline was determined by the method described in the preceding paper (16).

<div align="center">TABLE II</div>

<div align="center">*Adsorption of Rat Liver Phospholipid on MgO and Elution by Methanol*</div>

For a description of the procedure, see the text.

Extract	Sample No *	Amount of MgO used for adsorption	Phospholipid added to MgO			Phospholipid eluted by methanol			
			Choline	P	Moles choline / Moles P	Choline	P	Moles choline / Moles P	Recovery of choline phospholipid
		gm	mg	mg		mg	mg		per cent
A	1	1.0	3.35	1.49	0.58	3.36	0 96	0.90	100
B	2	1.0	3.43	1.47	0.60	3.42	0 97	0.90	100
	3	1.0	3.50	1.51	0.59	3.47	0 99	0.90	99
C	4	1.0	3 36	1.52	0 57	3.25	0 96	0.87	97
	5	1.75	3.30	1.52	0.56	3 36	0.96	0.90	98
	6	1.75	3 40	1.54	0.57	3 25	0.90	0.93	96
	7	1 75	3.40	1.54	0.57	3 26	0.90	0.93	96
	8	1.75	3 40	1.54	0 57	3 30	0 85	1 00	97
	9	1.75	3 40	1.54	0.57	3.26	0.85	0.98	96
D	10	1.75	3 47	1.54	0.58	3 33	0.85	1 00	96
	11	1.75	3.47	1.54	0 58	3.27	0.86	0 97	94
	12	1.75	3.47	1.54	0.58	3 19	0 83	0.99	92
E	13	1.75	3.42	1.49	0.59	3 25	0.88	0.95	95
	14	1.75	3 42	1.49	0.59	3.28	0.88	0.95	96
	15	1.75	3 42	1.49	0.59	3 21	0 84	0 98	94

* The results for each sample represent the average of closely agreeing duplicate or triplicate analyses

Table II shows that the elution of choline-containing phospholipids by methyl alcohol is practically complete when 1.0 gm. of MgO is used for adsorption. However, under these conditions the choline to phosphorus ratio of the eluted phospholipids was about 0.90. Since the choline to phosphorus ratio in pure choline-containing phospholipids would be expected to be unity, the finding of a ratio of 0.90 may be taken to indicate that the methanol eluate contained an appreciable amount of some non-choline phospholipid.

In an attempt to find conditions that would yield higher ratios, changes

in the pH of the methanol and effects of increase of temperature were tested. No consistent differences were observed when elution was carried out at 50° instead of at room temperature. Changes in the pH of the methanol also failed to raise the choline to phosphorus ratio. But when the amount of MgO was raised to 1.75 gm., the choline to phosphorus ratio of the eluate was found to be close to unity (Table II). The recovery of choline in the eluate under these conditions was about 95 per cent.

Adsorption of Phospholipid from Methanol by MgO—In the above experiments the phospholipids were first completely adsorbed from a petroleum

TABLE III

Adsorption of Liver Phospholipids Directly from Methanol

For a description of the procedure, see the text.

Extract	Sample No *	Phospholipid from liver of	Amount of MgO used for adsorption	Phospholipid added to MgO			Phospholipid that remained in methanol			
				Choline	P	Moles choline / Moles P	Choline	P	Moles choline / Moles P	Choline phospholipid recovered in methanol
			mg	*mg*	*mg*		*mg*	*mg*		*per cent*
F	1	Rat	1.0	3 51	1 51	0.59	3.49	0.99	0 90	99
G	2	"	1 75	3 42	1 49	0.59	3 36	0 88	0 98	98
	3	"	1.75	3 42	1 49	0.59	3 25	0 85	0 98	95
	4	"	1.75	3.42	1.49	0.59	3 30	0 91	0.93	96
H	5	Dog	1.5	3 56	1 55	0.59	3 38	0.93	0 93	95
	6	"	1 75	3 56	1 55	0 59	3 22	0.86	0 96	90
	7	"	1 75	3 56	1 55	0.59	3 25	0.88	0 95	91
J	8	"	1 75	3 46	1 51	0 59	3 14	0.82	0 98	91
	9	"	1 75	3 46	1.51	0 59	3 13	0 82	0 98	91

* The results for each sample represent the average of closely agreeing duplicate or triplicate analyses.

ether solution and elution of the choline-containing phospholipid carried out with methanol. This raised the question whether the separation of the two types of phospholipids could be accomplished by first dissolving the phospholipids in methanol and then treating this methanol solution with MgO. In order to test this possibility, the following experiments were carried out.

A measured volume of a petroleum ether extract of liver containing approximately 6 mg. of phospholipid P was evaporated just to dryness under reduced pressure in a CO_2 atmosphere and the residue dissolved in methanol. Under such conditions all phospholipids were dissolved by the methanol. This was shown by phosphorus and choline measurements. The methanol solution was made to 100 cc., and 25 cc. aliquots containing approximately 0.060 mg. of phospholipid P per cc. were added to 1.75 gm. of MgO in 50 cc.

centrifuge tubes. The mixtures were allowed to stand for 25 to 30 minutes with frequent stirring and then centrifuged. The methanol was poured off and the MgO washed twice with 25 cc. portions of fresh methanol. The combined methanol supernatants were made up to 100 cc. and aliquots taken for phosphorus and choline determinations. The results are recorded in Table III, which shows that the separation by this procedure is just as complete as when the phospholipids are first adsorbed from a petroleum ether solution and then eluted with methanol.

One of the difficulties encountered was a cloudiness of the methanol solutions which appeared after centrifuging the MgO-methanol mixtures. The first methanol supernatant was usually clear, but the supernatants from the two methanol washings were usually cloudy. It was found that the presence of a small amount of NaCl in the methanol prevented the solution from becoming turbid after centrifugation and did not affect the recovery of choline or the choline to phosphorus ratio. For this reason methanol containing approximately 5×10^{-3} M NaCl is used for the two washings of the MgO.

DISCUSSION

The method of separation of choline-containing from non-choline-containing phospholipids described here is both convenient and reasonably accurate. When a methanol solution of liver phospholipid was treated with MgO under the conditions specified above (1.75 gm. of MgO), the average choline to P ratio of the unadsorbed phospholipid was greater than 0.96. This may be taken to mean that practically all the phospholipids remaining in the methanol contain choline and therefore represent a mixture of lecithin and sphingomyelin. That a small amount of choline-containing phospholipid may remain behind with the MgO is suggested, however, by the finding that the recoveries are usually less than 100 per cent (Tables II and III).

The choline to P ratio determined on the phospholipid mixture before adsorption on MgO gives the fraction of the total liver phospholipids which is choline-containing. Thus the data in Tables II and III show that an average of 58 per cent of the phospholipid of the liver in the dog and rat contains choline. This value is in good agreement with that obtained by Artom and Fishman, who used the enneaiodide method for the determination of choline (13). These workers found that 60 per cent (average) of the phospholipid of the liver of rats fed a stock diet contained choline.

The work of previous investigators suggests that a separation of sphingomyelin from lecithin might be effected in the present method by precipitating the sphingomyelin as the reineckate from the methanol solution of the choline-containing phospholipids. In our hands this method has not yet yielded satisfactory results.

The application of this procedure to the separation of choline-containing from non-choline-containing phospholipids in tissues other than the liver is at present under investigation.

SUMMARY

A method for the separation of choline-containing from non-choline-containing phospholipid of the liver is described. The method is based on the discovery that phospholipids are completely adsorbed on MgO and that under certain conditions methanol elutes only the choline-containing phospholipids.

BIBLIOGRAPHY

1. Hevesy, G , and Hahn, L , *K. Danske Vidensk. Selsk Biol. Medd* , **15,** 5 (1940)
2. Chargaff, E , *J. Biol Chem* , **128,** 587 (1939).
3. Folch, J , *J Biol. Chem* , **146,** 35 (1942).
4. Kirk, E., *J. Biol. Chem.*, **123,** 623 (1938).
5 Sinclair, R. G., and Dolan, M , *J. Biol. Chem* , **142,** 659 (1942)
6 Ramsay, W. N. M , and Stewart, C P , *Biochem J.*, **35,** 39 (1941).
7. Thannhauser, S. J., and Setz, P., *J Biol Chem* , **116,** 533 (1936)
8 Thannhauser, S J., Benotti, J., and Reinstein, H , *J Biol. Chem.*, **129,** 709 (1939)
9. Erickson, B. M , Avrin, I , Teague, D. M , and Williams, H. H., *J. Biol. Chem* , **135,** 671 (1940)
10. Haven, F. L., and Levy, S. R., *J. Biol. Chem.*, **141,** 417 (1941)
11. Hunter, F. E., *J. Biol. Chem.*, **144,** 439 (1942)
12. Marenzi, A. D., and Cardini, C E., *J. Biol Chem* , **147,** 371 (1943)
13. Artom, C., and Fishman, W. H., *J. Biol. Chem* . **148,** 405 (1943)
14. Blix, G , *Biochem. Z* , **305,** 129 (1940).
15. King, E. J., *Biochem. J.*, **26,** 292 (1932).
16. Entenman, C , Taurog, A., and Chaikoff, I L , *J Biol Chem.*, **155,** 13 (1944).

THE EFFECT OF VITAMIN C ON THE DETERMINATION OF SULFANILAMIDE

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Because the therapeutic effectiveness of sulfanilamide is dependent on the attainment of an optimum concentration of the non-acetylated (free) drug in the blood, it is desirable to make routine determinations of the free sulfanilamide present in the blood of patients undergoing treatment (1, 2) Despite the fact that there have appeared reports dealing with combined ascorbic acid-sulfonamide therapy (3–8), there is nothing in the literature pertaining to the effect of vitamin C on the determination of sulfanilamide by the method of Bratton and Marshall (9). Yet it is shown by the works of several authors that large amounts of vitamin C may appear in the blood as a result of the administration of massive doses of ascorbic acid. In fact, blood levels as high as 25 mg. per cent have been reported in humans.

This investigation was undertaken to determine whether vitamin C interferes with the determination of sulfanilamide.

EXPERIMENTAL

The method used for the determination of sulfanilamide was that of Bratton and Marshall (9). Determinations of reduced vitamin C were made by the methods of Farmer and Abt (10).

Both *in vivo* and *in vitro* experiments were run.

For the *in vivo* experiments, blood was obtained by cardiac puncture from guinea pigs which were lightly anesthetized with ether. The animals used were of both sexes and weighed approximately 275 to 425 gm. The sulfanilamide was given by mouth by means of a rubber catheter attached to a suitable syringe. The drug was given as a 15 per cent water suspension in single doses of 323 mg. of sulfanilamide per kilo of guinea pig; and doses were administered at 0, 3, 7, 11, 15, and 19 hours (11). When ascorbic acid was given, it was administered intraperitoneally concurrently with the sulfanilamide dosage given *per os*. Blood samples were obtained 20 hours after the initial dose was given, and the samples were prepared at once for the determinations of vitamin C and sulfanilamide.

The following groups were given the sulfanilamide treatment: guinea pigs on normal diets, forty-six animals; 20 hours fasting, twenty-eight

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animals; vitamin C depletion for 10 to 13 days, twelve animals; parenteral injections of 2.5 mg. of ascorbic acid, six animals; and injections of 10 mg. of ascorbic acid, five animals.

In the *in vitro* experiments 10 mg. per cent of sulfanilamide were used throughout, except as noted in Table III.   The sulfanilamide-ascorbic acid solutions were made by adding to 5.0 cc. of the 20.0 mg. per cent stock solution of sulfanilamide used as the standard 5.0 cc. of a solution of ascorbic acid in trichloroacetic acid.   In the final sulfanilamide-ascorbic acid solution, the percentage of trichloroacetic acid was the same as that in the blood filtrates of Bratton and Marshall (9).   The coupling agent (N-1-naphthylethylenediamine dihydrochloride) was added after the final sulfanilamide-ascorbic acid solution had first been diluted 1:10.

Ascorbic acid solutions were oxidized by heating a distilled water solution of the vitamin under a reflux on a water bath for 1 hour.   The ascorbic acid oxidized in this manner was compared with the reduced form of ascorbic acid in regard to their effects on the determination of sulfanilamide.

Solutions of reduced ascorbic acid and sulfanilamide and solutions of oxidized ascorbic acid and sulfanilamide prepared as previously described were refluxed in a water bath at approximately 100° for 1 hour.   After the solutions were cooled and readjusted to the prerefluxing volume by the addition of distilled water, the free sulfanilamide was determined.

Sulfanilamide solutions alone when refluxed in a water bath at 100° for 1 hour gave complete recovery within the limits of error ($\sigma = 2.5$ per cent) previously found in this work.

All experiments were performed in duplicate.

DISCUSSION

Although different conditions in regard to the vitamin C level of the blood were found or were produced in the animals, there was no correlation between the amount of free or of conjugated sulfanilamide in the blood and the vitamin C content of the plasma (*cf.* Table I).

Inasmuch as the vitamin C in the blood was consistently very low at the time that the blood was withdrawn for the purpose of making tests for both sulfanilamide and vitamin C (*cf.* Table I, Column 2), it was thought that perhaps the sulfanilamide interfered with the determination of the vitamin, or caused a change in the *in vivo* metabolism of the vitamin.   *In vitro* tests showed, however, that sulfanilamide does not interfere in the determinations of ascorbic acid.   The low content of the vitamin in the blood can be explained by the fact that within 3 hours after the sulfanilamide has been administered the animals no longer take nourishment voluntarily.   It has been shown by Karel and Chapman (12) that after 20 hours fasting in guinea pigs, the blood level of vitamin C drops to low values approximating

those of Table I, Column 2. Consequently, although the drop in plasma ascorbic acid values may be the result of increased excretion or of metabolism of the vitamin attributable to the effect of the sulfanilamide (13–15),

<div align="center">TABLE 1</div>

Vitamin C Content and Free and Total Sulfanilamide Content of Blood Plasma of Normal Guinea Pigs after Six Doses of Sulfanilamide

15 gm. of sulfanilamide were suspended in 100 cc. of water. The dose was 32.3 mg. of sulfanilamide per 100 gm. of guinea pig by mouth. The schedule of dosage was at 0, 3, 7, 11, 15, and 19 hours. The weight of the guinea pigs was 272 to 432 gm. The date of the experiment was March 6, 1940.

Vitamin C prior to sulfanilamide dosage	20 hrs after 1st dose of sulfanilamide		
	Vitamin C	Free sulfanilamide	Total sulfanilamide
(1)	(2)	(3)	(4)
mg per cent	*mg per cent*	*mg per cent*	*mg per cent*
0.29	0.01	33.0	40 8
0 08	0 00	34.8	53.4
0.10	0.00	44.2	60 6
0.44	0 00	33.7	51.0
0.05	0.00	27.3	45.8
0.23	0.00	27.9	43.4
0 17	0.00	19 0	26.6
0.30	0.00	36.9	44.2
0.17	0.00	20.0	29.6
0.11	0.01	19.5	31.2
0.17	0.00	32.8	40 6
0.12	0 00	17.3	28.8
0.00	0 00	28.4	50.5
0.10	0.00	25 8	28.8
0.19	0 00	38 0	43.5
0.26	0.00	30.0	39.9
0.07	0 00	24.7	44.4
0.23	0 00	36.4	40.0
0.00	0 00	25 5	26.5
0.09	0.00	30 0	54.1
0.11	0 00	59 7	77.9
0.08	0.00	62 5	80.5
0 35	0 05	35 1	36.8
0.22	0 02	42.1	34.5
0.35	0.07	33.6	40.7
0.23	0 03	20.4	22.4

nevertheless, the low blood vitamin C values can be explained by the lack of vitamin C intake by the guinea pigs.

From the *in vitro* experiments, it was found that there is no interference with the determination of the drug when a solution contains 10.0 mg. per

cent of sulfanilamide and up to 3.0 mg. per cent of the reduced form of ascorbic acid.   Above 3.0 mg. per cent, reduced ascorbic acid in the presence of 10.0 mg. per cent of sulfanilamide interferes with the sulfanilamide determination increasingly the larger the quantity of the vitamin present.

TABLE II

In Vitro Experiments Showing Percentage of Sulfanilamide (10 Mg. Per Cent) Recovered in Presence of Varying Amounts of Vitamin C

Reduced vitamin C	Ratio of vitamin C to sulfanilamide	Sulfanilamide recovered	Oxidized vitamin C	Sulfanilamide recovered	Reduced vitamin C refluxed with sulfanilamide	Sulfanilamide recovered	Oxidized vitamin C refluxed with sulfanilamide	Sulfanilamide recovered
mg per cent		per cent	mg per cent	per cent	mg per cent	per cent	mg per cent	per cent
0 125	1:80	95 2						
0 25	1:40	97 4						
0 50	1:20	96.3			0 50	92 9		
0 75	1:13	95 0						
1 00	1:10	97 7			1 00	96 0		
1 25	1:8	97 7						
1 50	1:7	96 5						
1 75	1:6	98 0						
2 00	1:5	98 0			2 00	90 0		
2 25	1:4	97 5						
2 50	1:4	99 4						
2.75	1·4	97 4						
3 00	1:3	99 8			3 00	83.1		
5 00	1:2	93 3			5 00	77 8		
10 00	1:1	91 6	10 00	93 8	10 00	76 4		
15 00	3:2	92 0						
20 00	2:1	89 8	20 00	93 4	20 00	68 3	20 00	75.6
25 00	2 5:1	85 2						
30 00	3:1	84.2	30 00	88 9	30 00	62 2	30 00	66 3
40 00	4:1	77 3	40 00	73 2	40 00	57 6	40 00	56 7
50 00	5:1	71 5	50 00	63 1	50 00	56 6	50 00	50 7
100 00	10:1	56.1	100 00	65 0				
150 00	15:1	49 6						
200 00	20:1	24 5						
250 00	25:1	Trace						
300 00	30:1	0 0						

Interference with the color production is proportional not to the quantity of vitamin C present, but to the ratio of ascorbic acid to sulfanilamide (Tables II and III).

When the reduced form of the vitamin is heated with sulfanilamide in a water bath for 1 hour under a reflux, there appears to be a reaction between the ascorbic acid and the sulfanilamide.   Evidence for this lies in the facts

that the percentage of sulfanilamide recoverable is considerably reduced (Table II), that complete recovery can be obtained when sulfanilamide is heated alone, that if the ascorbic acid solution is first oxidized by heating in a water bath for 1 hour under a reflux and is then added to the sulfanilamide solution the recovery of sulfanilamide (which has *not been heated* with the oxidized vitamin) is approximately equal to the amount recoverable when solutions of sulfanilamide and reduced ascorbic acid (unheated) are tested, and that heating the sulfanilamide with previously oxidized ascorbic acid (Table II) gives results not appreciably different from those obtained when the reduced ascorbic acid is heated with the drug.

These latter facts are particularly of interest in regard to the determination of the total amount (acetylated plus non-acetylated) of sulfanilamide in solutions in which either the oxidized or the reduced form of vitamin C

TABLE III

Effect of Ratio of Vitamin C to Sulfanilamide on Recovery of Sulfanilamide

Sulfanilamide	Reduced vitamin C	Ratio of vitamin C to sulfanilamide	Sulfanilamide recovered
mg per cent	*mg per cent*		*per cent*
1.0	1.00	1:1	90.4
1 0	2 00	2:1	86.3
1 0	3 00	3:1	84 0
2.0	2 00	1:1	96 3
2 0	3.00	3:2	95.3
2.0	4 00	2:1	85.6
50 00	50 00	1:1	93 7
50 00	150 00	3:1	76 9

may be present, especially since less than 3.0 mg. per cent of ascorbic acid in the presence of 10.0 mg. per cent of sulfanilamide causes interference in the recovery of the sulfanilamide. This amount of ascorbic acid, it will be recalled, does not interfere in the determination of the non-acetylated sulfanilamide when the latter is present in the amount of 10.0 mg. per cent.

With respect to the effect of oxidized ascorbic acid on the determination of free sulfanilamide and on the determination of combined sulfanilamide, it is seen from Table II that results obtained with the oxidized form of the vitamin do not differ significantly from those obtained with the reduced form.

SUMMARY

1. Both the oxidized and the reduced forms of ascorbic acid interfere with the determination of sulfanilamide by the method of Bratton and Marshall (9) when the ratio of ascorbic acid to sulfanilamide is 1:2 or

greater.   Complete inhibition of the reaction does not occur, however, until the ratio reaches a value of 25:1 or greater (250 to 300 mg. per cent of ascorbic acid in the presence of 10.0 mg. per cent of sulfanilamide).

2. Heating the ascorbic acid and the sulfanilamide together in a water bath for 1 hour under a reflux results in a lowering of the amount of sulfanilamide recoverable even when the ratio of ascorbic acid to sulfanilamide is lower than 1:3.

BIBLIOGRAPHY

1. Mellon, R. R., Gross, P., and Cooper, F. B., Sulfanilamide therapy of bacterial infections, Springfield (1938).
2. Long, P. H., and Bliss, E. A., Clinical and experimental use of sulfanilamide, sulfapyridine and allied compounds, New York (1939).
3. Locke, A., Locke, R. B , Bragdon, R. J., and Mellon, R. R., *Science*, **86,** 228 (1937).
4. Cooper, F. B., and Gross, P., *Proc. Soc. Exp. Biol. and Med.*, **36,** 774 (1937).
5. Dainow, I., *Ann. mal. vénér.*, **34,** 513 (1939).
6. Locke, A., Main, E. R , and Mellon, R. R., *J. Immunol.*, **36,** 183 (1939)
7. Locke, A., *J. Immunol.*, **36,** 159 (1939).
8. Steinbach, M. M., and Duca, C. J., *Proc Soc. Exp Biol. and Med.*, **44,** 133 (1940)
9. Bratton, A. C., and Marshall, E. K , Jr , *J. Biol. Chem* , **128,** 537 (1939).
10. Farmer, C. J., and Abt, A. F , *Proc. Soc. Exp. Biol. and Med.*, **32,** 1625 (1935); **34,** 146 (1936).
11. Armstrong, A. R., and Thompson, J. L., *J. Lab. and Clin. Med.*, **24,** 1203 (1939).
12. Karel, L., and Chapman, C. W., *J. Nutr.*, in press.
13. Samuels, L. T., Ritz, N D., and Poyet, E B., *J. Pharmacol. and Exp. Therap* , **68,** 465 (1940)
14 Farmer, C. J , Abt, A F , and Aron, H C. S., *Proc. Soc Exp. Biol. and Med.*, **44,** 495 (1940).
15. Dainow, I., and Zimmet, D., *Compt. rend Soc. biol* , **130,** 846 (1939).

STUDIES ON KETOSIS

XXIII. THE METABOLISM OF ISOTOPIC TRIBUTYRIN AND PARTIALLY HYDROGENATED LINSEED OIL *

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The fate of tributyrin and butyric acid in intermediary metabolism is an unsolved problem. Tributyrin is readily digested and absorbed. Davis (1) found an average digestibility in rats of 86.9 per cent, while Deuel and Hallman (2) found that it was most rapidly absorbed of any of the short chain triglycerides. The absorption also has been found to be independent of the activity of the cortical hormones in distinction to the dependence of the fats whose constituent fatty acids are not water-soluble (3). Sodium butyrate is also as readily absorbed in normal rats (4) as in adrenalectomized salt-treated animals (3).

Eckstein (5) showed that absorbed tributyrin is not deposited in the depot fat of the rat. There is also no evidence that it may be converted to carbohydrate in the animal body when fed as sodium butyrate (6) or as the ethyl ester (7), but the glycerol of tributyrin does have glycogenic action (8). It has been considered by some that it may be changed to longer chain fatty acids and deposited as the triglycerides of such fatty acids. Rittenberg, Schoenheimer, and Evans (9) seem to have disposed of this possibility by demonstrating that the deuterium of deuterobutyric and deuterocaproic acids was completely converted to deuterium oxide within 8 hours after being fed. These authors point out that this may indicate a complete oxidation of these acids or a transfer of the deuterium during the conversion of the residue to other substances. However, the administration of deuterium oxide to rats fed an excess of ordinary butyric acid did not result in the deposition of any deutero compounds in the fat depots, a result which is believed to prove that the butyric and caproic acids are not transformed to such fats.

The present experiments were undertaken to obtain further evidence as to the fate of tributyrin and to compare its behavior with that of a natural triglyceride which is composed exclusively of long chain fatty acids.

EXPERIMENTAL

After the feeding of the isotopic fats to rats fasted 24 hours, the D_2 distribution at various periods up to 112 hours was followed in the lumen and

wall of the gastrointestinal tract, the tissue fat, the defatted tissue, and as D_2O in the tissue fluid and urine. The tributyrin was administered by stomach tube in a single dose and the hydrogenated linseed oil was given in divided doses. The animals were placed in metabolism cages for the collection of the urine and the stock diet was given to those animals on which the test was continued longer than 24 hours. Any feces were collected and analyzed separately or with the gastrointestinal contents. At the termination of the experimental period, the animals were anesthetized with amytal. In the experiments for periods shorter than 60 hours, the gastrointestinal tract was removed, weighed, and the contents flushed with ether. The deuterium content of the fat in this extract was determined separately. The gastrointestinal tract was dried in the vacuum oven. The remaining carcass was put through a meat grinder, the hashed tissues being passed through two additional times to insure uniformity, and an aliquot taken. This was dried at 50° in a vacuum oven to constant weight. The water removed was condensed by passing through a U-tube immersed in ethyl alcohol in a metal can surrounded by solid carbon dioxide. That the tissue water was quantitatively collected by this means was demonstrated by the fact that no increase in weight was ever found in the calcium chloride tube placed between the vacuum pump and the condensing tube. The tissue water was weighed, purified, and analyzed for deuterium. The dried tissue and intestines were extracted separately with ether for 16 hours in a Soxhlet apparatus. The lipids obtained from the ether extracts and the fat-free tissues were analyzed separately for deuterium. In some cases the fat-free tissue was powdered by being passed through a Wiley mill. This insured a greater homogeneity of the sample but greater difficulty in purification of the water obtained after combustion was experienced than when the coarser dried and defatted tissue was employed for burning without the final grinding. The removal of the organic contaminants in the water in the former case required repeated treatment with alkaline permanganate and chromic acid. However, we have found that this purification may be hastened by oxidation with potassium persulfate according to the procedure of Osborn and Werkman (10). The isotope analysis was made by the float method as employed earlier (11).

The hydrogenated linseed oil was prepared by an 82 per cent saturation of a sample of linseed oil with deuterium, with a platinum oxide catalyst. The iodine number of the original linseed oil was reduced from 192.1 to 35.3. For the feeding tests a mixture of 3 parts of the isotopic oil was mixed with 7 parts of untreated oil. This gave a sample liquid at body temperature which was tolerated by the rat.

Isotopic tributyrin was synthesized by the saturation of ethyl crotonate (prepared by the method of Florence (12)) with deuterium, transformation

to deuterobutenyl chloride by the procedure of Brown (13), and condensa-
tion with glycerol according to the method of Black and Overley (14).
The purity as determined by saponification was 99.3 per cent, and the
refractive index was 1.4350 compared with a value of 1.4359 for ordinary
tributyrin; also the tributyrin gave a volatile acid content which was 97
per cent of the theoretical. However, the deuterium content was only 75
per cent of the theoretical. It seems probable that a partial exchange with
hydrogen, due to contamination with crotonic acid, occurred during
saturation of the ethyl crotonate.

The deuterium content of the volatile fat of the lipid fraction of the
rat fed tributyrin 6 hours previously was determined on the distillate as
follows: The saponified fat was acidified with H_2SO_4, 200 cc. of water were
added, and this was distilled. The distillation was repeated after the
addition of a second 200 cc. portion of water, and the distillates were
combined. The distillate was concentrated to about 150 cc. after being
made alkaline to prevent a distillation of the volatile acids. An aliquot
was oxidized with potassium persulfate and the deuterium content was
determined by the usual float method.

Rats from our stock colony which had previously received our stock diet
and which weighed approximately 100 gm. were used.

Results

The analyses of the tissues and urine of five rats at various periods after
they had received tributyrin and of four rats at several intervals after they
were fed partially hydrogenated linseed oil are summarized in Table I.
Control tests were also made on the recovery of these fats when known
amounts were added to the hashed tissues of the rats, as well as on their
recovery from the gastrointestinal tract when it was flushed out directly
after the fats were administered by stomach tube.

DISCUSSION

The absorption of tributyrin proceeds much more rapidly than that of
the partially hydrogenated linseed oil. Within 6 hours it was 95 per cent
complete; on the other hand only 30 per cent of the hydrogenated linseed oil
was absorbed in 12 hours, although the absorption was completed in 24
hours. The slower rate of disappearance must be partly ascribed to the
larger quantity of the oil administered and to the fact that it was given in
divided doses.

The extent of incorporation of the administered fats into the body fat is
also markedly different with the two fats studied. Thus, the maximum
accounted for in the tissue lipids in the tributyrin experiments was 12 per
cent at 12 hours, while the highest value recorded after the hydrogenated

linseed oil was 53 per cent at the 24 hour period.   Only 2 per cent of the tributyrin was present in the tissue lipids at 36 hours and it was completely absent in the 60 hour test and thereafter; on the other hand, after the

TABLE I

Fate of Deutero Fats When Fed to Fasted or Fed Albino Rats

Time after feeding	D₂ content of amount fed	Per cent of administered deuterium recovered								Remarks
		Gut content	Gut wall	Tissue lipid	Fat-free tissue	Tissue fluid (1)	Urine (2)	Sum of (1)+(2)	Total	
						Tributyrin				
hrs.	*mg. D₂O*									
0	58.2			37.1	55.0	6.4			98.5	Added to hashed tissue
0	72.8			51.0	39.9	6.7			97.6	
0	85.0	95.1	6.4						101.5	Recovered from gut
6	72.8	4.4	1.0	9.3*	19 6	65 2	2 2	67.4	101.7	
12	72.8	0.0	0.0	12.0	7.1	73.2	6.2	79.4	98.5	
36	72.8			2.1	4.0	77.3	22 1	99 4	105.5	
60	72 8	13 8†	0 0	0 0	0 0	58 8	27 7	86 5	100.3	
						68.0	32 0	100 0		Calculated on basis of fat absorbed
107	72.8			0.0	0 0	40.7	57.4	98.1	98.1	
						Partially hydrogenated linseed oil				
0	27 0			98.0	0.0				98.0	Added to hashed tissue
0	27.0	91 0	5 9						96.9	Recovered from gut
12 (4)‡	57 4	63.3	6 4	9 8	4 6	17 8	4.6	22 4	106.5	
24 (6)‡	57.4	1.1	1.6	52 6	17 9	19.7	1.6	21.3	95 8	
48 (6)‡	57.4	0.0		20.2	24 9	42.0	14.3	56.3	101 4	
112 (6)‡	57.4			19.3	6.1	47.2	30.1	77.3	102.7	

* This was entirely in the distillable fraction, the value on the distillate being 9.2.

† Deuterium lost in feces.

‡ Given in divided doses.   The figure in parentheses indicates the period in hours after the first dose when the second dose was administered.

feeding of the linseed oil 20 per cent was still present in the tissue lipids after 48 hours and no further decrease occurred in over 4½ days (112 hours).

A comparison in the rate of metabolism is also afforded by the extent of conversion of the isotopic fat to heavy water.   This first accumulates in the

tissue fluid, gradually reaching a maximum, and then it slowly drops, corresponding to the rate at which it continues to be formed and at which it is excreted in the urine. Based on such conditions within 6 hours, 67 per cent of the tributyrin has been completely destroyed. On the other hand, only 21 per cent of the hydrogenated linseed oil was metabolized in 24 hours, 56 per cent in 48 hours, and 77 per cent in 112 hours. As has been noted by other investigators, the rate of excretion of deuterium oxide from the tissue fluid is exceedingly slow.

The deuterium remaining in the tissue after exhaustive extraction with diethyl ether is in all probability present in soaps, although the possibility exists that the isotope might have been set free and exchanged with the hydrogen on other compounds. The ease of saponification of tributyrin *in vitro* as well as *in vivo* is well known and this would seem to offer an explanation for the large fraction which was unextractable with ether. In recovery experiments with tributyrin added to hashed tissues, a conversion of 40 to 55 per cent of the isotopic lipid to material not extractable by ether occurred on drying. On the other hand, in the recovery tests with the hydrogenated linseed oil, 98 per cent of the deuterium remained in the lipid fraction on drying.

The present experiments confirm the work of Eckstein (5) which showed that tributyrin is not stored in the depot fat over extended periods of time. There is, however, a temporary storage of small amounts of isotopic tributyrin or butyric acid which amounts to 12 per cent of the amount fed at 12 hours, and 2 per cent still was present at 36 hours. That the deuterium so bound is still combined with butyrate is indicated by the demonstration in the 6 hour test that the distillable fatty acids from the saponified tissue fat accounted for 100 per cent of the deuterium. On the other hand, we have no evidence as to whether such a butyrate residue is present as tributyrin, in a mixed triglyceride, or as the free acid. Because of the ready susceptibility of tributyrin to hydrolysis, it will probably only be possible to settle the question of whether the butyrate is free or combined with glycerol by immediate freezing of the intact animal, grinding the frozen tissue, and drying it in a lyophile apparatus.

The experiments also confirm Rittenberg, Schoenheimer, and Evans (9) in indicating that the butyrate fragments are not directly used for interconversion to long chain fatty acids. There is still the remote possibility that simple derivatives from butyrate might play a rôle in the formation of specific long chain fatty acids. If this were the case, it could best be settled by the administration of butyrate containing isotopic carbon.

The recovery of the deuterium fed varied from 96 to 106 per cent. This would seem to indicate the accuracy of the experimental technique. Also the fact that blank values were obtained on the gut contents, gut wall,

tissue fat, or fat-free tissue in control tests or in samples at prolonged periods after the feeding of isotopic fats would seem further to confirm the accuracy of the results.

SUMMARY

Isotopic tributyrin and linseed oil partially hydrogenated with deuterium were fed to rats and the distribution was noted in the lumen of the gastro-intestinal tract, gut wall, tissue fluid, tissue fat, fat-free tissue, and urine at several periods up to 112 hours.

Tributyrin was found to be much more rapidly metabolized than the oil, having almost disappeared from the tissue fat at 36 hours and completely at 60 hours. Partially hydrogenated linseed oil was still present in the tissue fat to the extent of 20 per cent of the amount fed after 112 hours. Moreover, the maximum amount of tributyrin in the tissue fat was 12 per cent of that administered, while the maximum value found in the tests with the isotopic oil was 53 per cent of the quantity fed.

On the basis of a single experiment, it would appear that the deuterium in the tissue fat after the feeding of isotopic tributyrin could be entirely accounted for in the distillable fraction. This would indicate that no direct conversion of tributyrin to long chain fat occurs. The experiments indicate a temporary storage of the butyrate molecule, but whether this is retained as tributyrin, as a component of a mixed triglyceride, or as butyric acid could not be ascertained.

BIBLIOGRAPHY

1. Davis, R. E., *J. Biol. Chem.* **88**, 67 (1930).
2. Deuel, H. J., Jr., and Hallman, L , *J. Nutr.*, **20**, 227 (1940).
3. Bavetta, L., Hallman, L , Deuel, H. J., Jr , and Greeley, P O , *Am J. Physiol.*, **134**, 619 (1941).
4. Deuel, H. J., Jr., Hallman, L., and Reifman, A G., *J. Nutr.*, **21**, 373 (1941).
5. Eckstein, H. C., *J. Biol Chem* , **81**, 613 (1929).
6. Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., and Cutler, C. H., *J. Biol. Chem.*, **112**, 15 (1935–36).
7. Deuel, H. J., Jr., Hallman, L F., Butts, J. S , and Murray, S., *J. Biol. Chem.*, **116**, 621 (1936).
8 Deuel, H J., Jr., Butts, J. S., Blunden, H., Cutler, C. H., and Knott, L., *J. Biol. Chem* , **117**, 119 (1937).
9. Rittenberg, D , Schoenheimer, R , and Evans, E. A., Jr., *J. Biol. Chem.*, **120**, 503 (1937).
10. Osborn. O L , and Werkman, C H., *Ind. and Eng. Chem.*, *Anal. Ed.*, **4**, 421 (1932).
11. Morehouse, M. G., *J Biol. Chem.*, **129**, 769 (1939).
12. Florence, G., *Bull. Soc chim.*, **41**, 440 (1927).
13 Brown, H. C., *J. Am. Chem. Soc.*, **60**, 1325 (1938).
14 Black, H C., and Overley, C. A., *J. Am. Chem. Soc.*, **61**, 3051 (1939).

CLEAVAGE OF CYSTATHIONINE BY AN ENZYME SYSTEM FROM RAT LIVER

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Cystathionine has been shown to serve in lieu of cystine in the diet of rats (1) and to be cleaved by liver tissue of rats with the formation of cysteine (2). These findings, together with the observation that considerable extra cysteine is produced when a mixture of homocysteine and serine is incubated with rat liver tissue, may be interpreted as indicating that methionine is converted to cystine through an intermediate formation of homocysteine and cystathionine (3) Additional support for this hypothesis has been gained from studies of the metabolism of compounds labeled with heavy isotopes. Stetten (4) has reported evidence indicating that the carbon chain of cystine is derived from serine and, more recently, du Vigneaud and coworkers[1] have demonstrated that the original carbon chain of methionine does not appear in the cystine formed from it by the white rat.

While these recent studies have done much to clarify the question of the major intermediary steps in the conversion of methionine to cystine, little or no knowledge has been gained as to the nature and mode of action of the enzymes involved in this conversion. An initial study on one of these enzymes is described in this report. The evidence presented may be interpreted as indicating that the cleavage of cystathionine is accomplished by a mechanism utilizing phosphate bonds high in energy. The system involved in the cleavage includes a heat-labile enzyme, a metallic ion, zinc or magnesium, and adenosine triphosphate. The high energy phosphate of adenosine triphosphate is dissipated coincident with the cleavage of cystathionine and the dissipated phosphate is found, not as inorganic phosphate, but as a phosphate compound of low energy, stable to acid hydrolysis. Little or no free ammonia is released during the cleavage and it would seem probable that the cleavage product, other than cysteine, is the phosphorylated hydroxyamino acid, the next higher homologue of serine or phosphohomoserine.

The net result of the series of reactions would be the exchange of the sulfhydryl group of homocysteine for the hydroxyl group of serine. Since the emphasis in these studies is on the shift of the sulfhydryl group, the

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[1] du Vigneaud, V., personal communication.

reaction may be described as "transsulfuration." The possibility that the process plays a metabolic rôle beyond the conversion of methionine to cysteine is interesting. An extension which may present an alternate partial explanation of the observations made by Stetten (4) is that serine and cysteine may be in equilibrium in so far as their carbon chains are concerned. Such an equilibrium would involve an intermediate formation of the symmetrical thio ether lanthionine. The finding that lanthionine, like cystathionine, is cleaved by rat liver tissue with the formation of cysteine[2] lends support to this hypothesis.

EXPERIMENTAL

Preparation of Enzyme Solution—Fresh liver tissue of rats was mixed in a Waring blendor at 0° with a 10-fold amount of physiological saline for 15

TABLE I

Reconstitution of System for Cleavage of Cystathionine

1.5 mg. of cystathionine (equivalent to 0.82 mg. of cysteine), 30 minutes digestion at 38° in 0.06 M bicarbonate, total volume 6 cc.

Digest	Extra cysteine	Yield
	mg	*per cent*
1 cc. crude extract	0 43	51
1 " enzyme preparation	0 07	9
1 " boiled crude extract	0.00	0
1 " " " " 1 cc enzyme preparation	0.35	43
1 " enzyme preparation, 0 001 M ZnSO₄	0.10	12
1 " " " adenosine triphosphate (0.22 mg. labile P)	0.21	26
1 cc. enzyme preparation, adenosine triphosphate, ZnSO₄	0.54	66

seconds. The mixture was then centrifuged at 15,000 R.P.M. for 30 minutes and the sediment discarded. The opalescent supernatant solution was placed in a cellophane bag and dialyzed at 0° for 48 hours against frequent changes of physiological saline. The dialyzed material was heated for 10 minutes at 55°, cooled immediately to 0°, and centrifuged at high speed to remove the precipitate. The supernatant solution, which contained 5 to 8 mg. of trichloroacetic acid-precipitable material per cc. of solution, was the enzyme solution used in these studies.

As shown in Table I, this material brought about little or no cleavage of cystathionine and was effective only in the presence of zinc ions and adenosine triphosphate or in the presence of boiled crude extract.

Technique of Digestion and Analysis—All the data reported were obtained

[2] Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., unpublished observations.

from digestions conducted in 0.06 M bicarbonate solution. The cysta-thionine,[3] zinc or magnesium salts, and adenosine triphosphate were dissolved in bicarbonate solution. The various solutions, including the enzyme preparation, were saturated with CO_2 and warmed to 38° before being mixed. A sample was removed at zero time and the mixture saturated with CO_2. The tubes were tightly stoppered and incubated at 38°. Samples were taken at various intervals, and the protein was precipitated by the addition of trichloroacetic acid. The protein precipitate was removed by centrifugation and the cysteine in the supernatant solution determined directly by the method of Sullivan and Hess (5). Recovery experiments were made to demonstrate that added cysteine could be recovered with little or no deviation from the theoretical values. Free ammonia was determined by a procedure of steam distillation in which the trichloroacetic acid supernatant was made alkaline with a borate-NaOH buffer. The inorganic phosphate, the phosphate released by an 11 minute and by a 30 minute hydrolysis in 1 N sulfuric acid at 100°, and the total phosphate were determined by the method of Fiske and Subbarow (6).

DISCUSSION

In Table I it is seen that the thoroughly dialyzed enzyme preparation was inactive unless either boiled crude extract or a combination of zinc ions and adenosine triphosphate was added. Experiments not included in Table I showed magnesium ions to be of the same order of efficacy as the zinc ions, whereas equivalent amounts of cupric and ferric ions were inhibitory. Adenylic acid or thiamine pyrophosphate would not replace the adenosine triphosphate when tested in this system. Thiamine pyrophosphate, however, did stimulate the action of a crude extract or of an incompletely dialyzed preparation.

The experiments described in Table II were conducted to determine the availability of adenosine triphosphate in the reaction. If the cleavage is pictured as a simple phosphorolysis in which phosphoric acid is added at the carbon-sulfur bond, both of the labile phosphates of the adenosine triphosphate might be expected to be available for the cleavage. If two phosphates are utilized, the addition of 7.1 micromoles of labile phosphate should lead to the production of 7.1 micromoles of extra cysteine. The cysteine production was found to be 4.0 micromoles or about half of the calculated amount. The addition of 21.3 micromoles of labile phosphate led to the production of 10.3 micromoles of cysteine. These results emphasize the fact that the extent of the reaction is dependent upon the amount of adenosine triphosphate in the system. They also indicate that

[3] The cystathionine was furnished for this problem by Professor V du Vigneaud of Cornell University Medical College.

only one phosphate of the adenosine triphosphate is available for the cleavage or, alternatively, two labile phosphates are required for the cleavage of 1 molecule of cystathionine.

From the results in Table III, in which the phosphate distribution is given, it is seen that only one acid-labile phosphate is utilized in the cleavage. For the production of 3.5 micromoles of cysteine 3.5 micromoles of

TABLE II

Dependence of Extent of Cystathionine Cleavage upon Amount of Adenosine Triphosphate

3 mg of cystathionine (equivalent to 13 6 micromoles of cysteine), 2 cc of enzyme solution, 0 001 M ZnSO₄, total volume 15 cc.

Labile phosphate added as adenosine triphosphate	Total cysteine		
	30 min	60 min	90 min
micromoles	*micromoles*	*micromoles*	*micromoles*
None	0 7	0 9	0 8
7 1	3 8	4 9	4 6
21 3	7 8	11 2	10 3

TABLE III

Phosphate Turnover during Cleavage of Cystathionine

3 mg of cystathionine, adenosine triphosphate (7 1 micromoles of labile P), 0 001 M ZnSO₄, 2 cc of enzyme solution, total volume 15 cc.

Time	Total cysteine	Phosphate distribution		
		Inorganic	Acid-labile*	Acid-stable
min	*micromoles*	*micromoles*	*micromoles*	*micromoles*
0	1 0	3 3	7 4	4 5
30	3 5	3 4	5 1	6 7
60	4 9	3 5	3 6	8 1
90	4 5	3 6	3 1†	8 5
Change	+3 5	+0 3	−4 3	+4 0

* Inorganic phosphate released by an 11 minute hydrolysis in 1 N H₂SO₄ at 100°
† Increased to 3 4 by 30 minutes hydrolysis in 1 N H₂SO₄ at 100°.

labile phosphate should be dissipated. The observed disappearance of 4.3 micromoles is in agreement with this hypothesis, especially when it is recognized that the total cysteine found at 90 minutes was less than that found at 60 minutes. The agreement at 60 minutes is, therefore, somewhat better. The decrease in the total cysteine after 60 minutes was a constantly observed phenomenon and was probably due to the destruction of cysteine

by the enzyme desulfhydrase[4] or to an oxidation of the cysteine to cystine In control experiments in which no substrate was added, the labile phosphate remained essentially unchanged during the 90 minute period of digestion.

The labile phosphate dissipated in the reaction was distributed between an inorganic fraction and an acid-stable fraction with the greater quantity being found in the latter fraction. No significant increase in ammonia was detected at the end of the 90 minute period and, although the carboxyl-amino nitrogen was not determined, it is probable that the amino groups remained intact during the cleavage of the thio ether linkage.

If the same mechanism of cleavage described for liver tissue of rats is valid in the intact animal, a definite relationship between this phase of the intermediary metabolism of an amino acid and the metabolism of carbohydrates is established. The high energy phosphate of adenosine triphosphate developed by the oxidative breakdown of carbohydrates would be utilized by the animal to carry out an essential metabolic transformation of one amino acid to another. The amino acids involved in the transformation would contribute to the energy needed in the transformation only in so far as they are precursors of carbohydrate or other materials utilized in the formation of phosphate bonds of high energy

SUMMARY

Cystathionine, a possible intermediate in the conversion of methionine to cystine by the animal organism, is cleaved with the formation of cysteine by an enzyme system derived from the liver of the rat through a mechanism involving a simultaneous transfer of the terminal phosphate group of high energy of adenosine triphosphate. It is suggested that the cleavage of the thio ether is effected by the addition of phosphate at the carbon-sulfur bond.

BIBLIOGRAPHY

1. du Vigneaud, V , Brown, G B , and Chandler, J. P., *J. Biol. Chem.*, **143,** 59 (1942).
2. Binkley, F , Anslow, W P , Jr , and du Vigneaud, V , *J. Biol. Chem.*, **143, 559** (1942)
3. Binkley, F., and du Vigneaud, V , *J Biol. Chem* , **144,** 507 (1942).
4. Stetten, D , Jr , *J. Biol Chem* , **144,** 501 (1942).
5. Sullivan, M X , and Hess, W C , *J. Biol. Chem.*, **116,** 221 (1936).
6 Fiske, C H , and Subbarow, Y , *J. Biol. Chem.*, **66,** 375 (1925).

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[4] The term desulfhydrase has been suggested by C. V. Smythe to describe the enzyme which removes hydrogen sulfide from cysteine.

STUDIES ON PITUITARY LACTOGENIC HORMONE

X. THE EFFECT OF A DETERGENT [*]

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It has been shown that the lactogenic hormone is not reduced in biological activity by treatment with urea if the urea is removed by dialysis before the bioassays (1). It is probable that urea denatures the hormone molecule and that the denatured protein reverts to its native state when the denaturing agent is removed. Since it is impossible to assay the hormone in urea solutions, it cannot be concluded that the hormone is active in such solutions.

The increase in relative viscosity may be used as an indication of denaturation in protein (2). In the presence of 3.0 M urea, the relative viscosity of lactogenic hormone is greatly increased. When the urea has been removed by dialysis, the original relative viscosity of the hormone is retained. The results are summarized in Table I. These experiments indicate clearly that a change of the hormone molecule occurs in the urea solution and that this change is a reversible process as judged by the viscosity measurements.

Since detergents are good denaturing agents (4) and since proteins can be assayed biologically without the removal of detergents, it was of interest to see whether or not the lactogenic activity of the hormone is retained in the presence of a detergent.

Fig. 1 shows the alteration in relative viscosity of lactogenic hormone in Nacconol[1] solutions. As is the case with urea, the relative viscosity of the hormone increases as the detergent concentration increases. When these solutions were assayed[2] in month-old squabs, the lactogenic activity was shown to be diminished (Table II).

It has been demonstrated in the case of insulin (5) and egg albumin (6) that a complex formation occurs between the detergent and the protein. The rise of relative viscosity of lactogenic hormone in Nacconol solution may be due to change in the molecular shape of the hormone or to a hor-

[1] Nacconol, N. R. S. F., a detergent of the sodium alkylarylsulfonate type, manufactured by the National Aniline Division, Allied Chemical and Dye Corporation.

[2] I am indebted to Dr. H. M. Evans and Dr. M. E. Simpson for the bioassay of the solutions.

mone-detergent complex formation.   Since attempts to remove the deter-gent from the hormone were unsuccessful, it is likely that a complex is formed between Nacconol and the hormone.

TABLE I

Relative Viscosity of Lactogenic Hormone in 3.0 M Urea at 25°

Lactogenic hormone	Concentration	Relative viscosity
	per cent	
In 3.0 M urea	0 57	1.058
	0 43	1 043
	0 21	1.022
After removal of urea by dialysis	0 60	1.029
	0 34	1.015
	0.26	1 011
In pH 7.0 phosphate buffer*	0.55	1 025
	0 39	1 020
	0 28	1 014

* Taken from Table I of Paper VIII (3).

TABLE II

Bioassays of Lactogenic Hormone in Month-Old Squabs

Concentration of Nacconol in pH 7 0 phosphate buffer	Total dose of lactogenic hormone	Route of injection	Crop reaction*	Estimate
per cent	*mg*			*i u per mg*
0	0 2 .	Intramuscular	+, +, +	30
	0 1	Subcutaneous	+, +, +	
2	1 0	Intramuscular	+, +, +, +, +	
	0.75	"	+, +, +	15
	0.40	"	−, +, +	
	0 20	"	−, −, −, −, −, −	
3 8	1 0	Subcutaneous	2+,2+,2+,3+,2+,4+	
	0.75	"	2+, +, 2+	5
	0 50	"	−, −, −, −	
3 8	2 0	Intramuscular	+, +, +	
	1 5	"	−, +, −	7
	1.0	"	−, −, −	
	0 5	"	−, −, −	

* The lactogenic activity was classified into one of four arbitrary groups ac-cording to the degree of proliferation in the crops, + being the minimal detect-able response and the maximal reaction 4+.

Previous experiments (7, 8) indicate that the biological activity of lactogenic hormone depends on the intrinsic structure of tyrosine and

amino groups in the molecule.   If the hormone-detergent complex forma-
tion occurs, the loss of lactogenic activity may be due to interactions be-
tween the detergent and either or both of these groups.

The methods for the measurement of viscosity and density have been
described in a previous paper (3).   The urea used was the c.p. grade with-
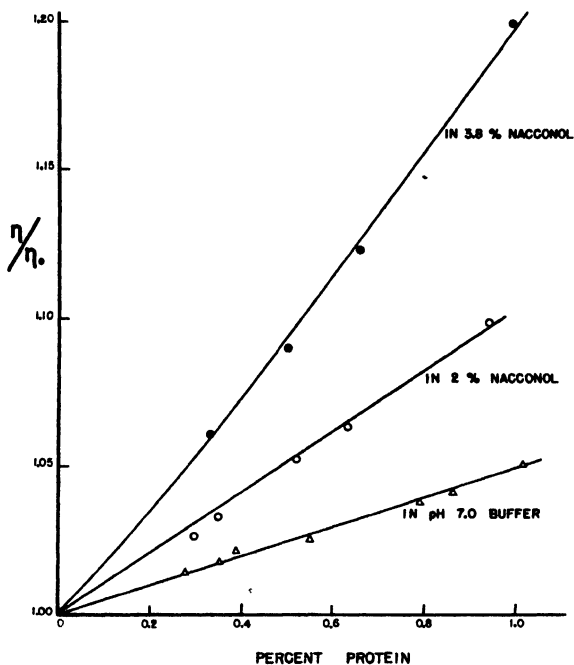
Fig. 1. Relative viscosity of lactogenic hormone plotted against the protein
concentration in weight per cent.

out further purification.   The detergent was also not further purified.
The lactogenic hormone used had been shown to be homogeneous in the
electrophoresis experiments and was prepared according to the method
described previously (9) from sheep pituitary.   The bioassay of the hor-
mone was carried out in month-old squabs as is usually done in this labora-
tory (9) and had a potency of 25 to 30 I.U. per mg.

SUMMARY

The relative viscosity of lactogenic hormone solutions in the presence of
urea and of a detergent (Nacconol) has been determined.   The increase of

viscosity and coincident lowering of biological activity of the hormone in detergent solutions have been demonstrated.

BIBLIOGRAPHY

1. Li, C. H , Lyons, W. R., and Evans, H. M., *J. Biol. Chem* , **140**, 43 (1941). •
2. Neurath, H., Cooper, G K , and Erickson, J. O., *J. Phys. Chem* , **46**, 203 (1942).
3. Li, C. H., *J. Biol. Chem* , **146**, 633 (1942).
4. Anson, M. L., *Science*, **90**, 256 (1939); *J Gen. Physiol* , **23**, 239 (1939)
5. Miller, G. L , and Andersson, K. J. I , *J. Biol Chem* , **144**, 475 (1942)
6. Lundgren, H P , Elam, D. W , and O'Connell, R A , *J Biol Chem* , **149**, 183 (1943)
7 Li, C. H., Simpson, M E , and Evans, H M , *Science*, **90**, 140 (1939).
8 Li, C. H , Lyons, W R., and Evans, H. M , *J Biol. Chem* , **139**, 43 (1941).
9 Li, C. H., Simpson, M E , and Evans, H M , *J Biol Chem.*, **146**, 627 (1942).

THE INFLUENCE OF SUPPLEMENTARY CASEIN, CYSTINE, AND METHIONINE ON LIVER LIPID CONTENT OF ADULT RATS

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In recent reviews on the relationship of choline and allied substances to lipid metabolism (1, 2) considerable space has been allotted to the question of the influence of amino acids and protein on liver lipid content. It has been generally observed that the accumulation of unusual amounts of "fat" that occurs in the livers of rats on a basal diet low in protein and high in fat (*i.e.*, a lipogenic diet) can be prevented by adding either free methionine or additional proteins containing methionine to the ration. When such a basal diet is supplemented with cystine, as the free amino acid, the level of liver lipid content is even higher than when the lipogenic diet is fed alone. The response obtained with methionine is currently ascribed to the presence of a labile methyl group, but divergent opinions have been given to account for the results obtained with protein. In the minds of Channon and coworkers (3) "either some other amino acid exerts lipotropic action or alternatively added methionine is incapable of exerting its full action in the absence of some other protein constituent." On the other hand, Eckstein and his coworkers at Michigan (4, 5) believe that the resultant effect of different dietary proteins on liver lipid content can be ascribed to their methionine and cystine contents. With the exception of threonine and hydroxyglutamic acid, all of the naturally occurring amino acids have been studied with respect to their influence on liver lipid content and none has been found to behave like cystine or methionine (5–7). Beeston and Platt (6) at one time reported that tyrosine exhibits some lipotropic action, but even this has recently been denied (7). Channon and associates (7) have embarked on a new method of attack by comparing the effect on liver lipid content of the addition of different fractions of casein hydrolysates to a lipogenic diet. Their observation that casein, completely hydrolyzed with sulfuric acid, is as effectively lipotropic as the original protein indicates that protein constituents concerned with this phenomenon are not significantly altered by the prolonged action of this mineral acid. In a

series of additional experiments native casein, the butanol-soluble fraction of completely hydrolyzed casein, and the butanol-insoluble portion of such a hydrolysate were added separately to a lipogenic diet (Diet G). The methionine, cystine, and tyrosine contents of these supplemented rations were equalized by the addition of the amino acids in the free form. Tyrosine was included because of their belief that it might, in conjunction with other protein constituents, influence liver lipid content. Their data show that a lipotropic action occurred when basal Diet G was supplemented in these three ways. The butanol-soluble fraction proved to be most effective, while the butanol-insoluble portion was least efficient. These observations led to the conclusion that the butanol-soluble fraction of hydrolyzed casein contains more of an additional amino acid which decreases liver "fat" content or less of one which accelerates liver lipid deposition. In other experiments Diet G was supplemented with (a) 15 per cent casein, (b) 15 per cent gelatin plus methionine, cystine, and tyrosine, and (c) methionine, cystine, and tyrosine. The amounts of the free amino acids added in (b) and (c) were sufficient to equalize their content in all three diets. All three of these supplemented rations proved to be lipotropic and the average value for liver lipid content was essentially the same in the three cases. Evidently the equalization of the content of the three amino acids in the diets was sufficient to produce the same degree of lipotropic action. These findings adequately support the view held at Michigan, and thus in the same investigation different methods of attack supply opposing answers to the problem. Treadwell, Groothuis, and Eckstein recently (8) observed that the liver lipid content of young rats on a basal lipogenic diet supplemented with methionine was significantly lower than that of young rats in which the methionine content of the ration was increased to the same extent as above by the addition of casein. These findings suggested that methionine in the free form is superior to casein so far as lipotropic action is concerned. However, in discussing this apparent complication Treadwell et al. (8) carefully pointed out that the inferior response obtained with casein might be ascribed to the greater increases in growth that occurred when the protein was used as the supplement. Larger amounts of newly formed tissue were undoubtedly laid down by these young rats on this high protein régime, and the additional methionine was thus required for the deposition of extra amounts of tissue protein. In view of this increased requirement, less methionine may have been available for lipotropic action. It was therefore suggested that it would be desirable to repeat these experiments with adult rats in the light of the belief that the protein requirement of the rat decreases with age. According to Hamilton (9) young rats require from 16 to 20 per cent of whole egg protein for maximum gains in body weight, whereas 4 per cent of this material is

sufficient for the maintenance of nitrogen equilibrium in mature rats (10) (weight 250 gm.). In addition, Mitchell and Carman have demonstrated (11) that, in contrast to the significant rise in the percentage of total lipids of the whole rat that accompanies increase in age, a small but definite fall in the per cent of total nitrogen occurs as rats become older. It is evident therefore that, as growth proceeds, the increases in weight can more and more be ascribed to the deposition of greater proportions of fat, and it should follow that with this increase in age less methionine needs to be requisitioned for growth and more should be available for other purposes. The investigation to be described was carried out with these ideas in mind.

Adult male rats (weight 250 gm.) were placed on a basal lipogenic diet supplemented with amino acids in the same manner as in the experiments with young rats referred to above (8). This basal diet consisted of 2 per cent agar, 5 per cent casein, 40 per cent lard, 5 per cent salt mixture (12), and 48 per cent glucose. In addition each rat received 1 dry yeast tablet (500 mg.) and 2 drops of cold liver oil daily. When the supplements of casein and the amino acids specified in Table I were added to the basal diet, equivalent amounts of glucose were withheld. Cystine was added to the diets in order to make these experiments comparable with our previous experiments with young rats (8). The experiments were terminated at the end of 3 weeks. The method for total lipids differed slightly from our regular procedure (14) in that the livers were first frozen in dry CO_2, and then pulverized in a metal crusher (15) prior to the usual extraction with alcohol and ether.

Table I contains a summary of the results obtained in this investigation as well as some of the data previously secured in this laboratory with young rats (8). The latter are included for the sake of comparison. It is evident that fatty livers can be produced when the basal ration is fed to either young or old rats. This is illustrated in Series I, in which the average liver lipid contents of the old and young rats are 24.2 and 23.6 per cent respectively. In the remaining experiments, significant differences were observed between the responses of the young and old rats to dietary changes. Thus in Series II, the average value for liver lipid content of the older rats (Group 3), receiving the basal diet supplemented with the free amino acids, is practically the same as the corresponding value for the older rats in Group 17 in which case the methionine content of the diet was increased by the addition of casein. On the other hand, the average value for liver lipids of the young rats in these two groups differed decidedly, the respective values for Groups 3 and 17 being 11.2 and 26.3 per cent. These observations are confirmed in Series III in which identical values are recorded for the adult rats in Groups 4 and 18, and marked differences are reported for the young in these groups. In Series IV the values 9.5 and

10.8 per cent for the adult rats in Groups 6 and 19 respectively compare well with each other, while those for the young (15.7 and 26.5 per cent) differ significantly.   In Series V, the values for the adults are again in close agreement with each other, whereas those of the young are appreciably different.

The data on change in body weight show that all of the rats on the supplemented diets gained in weight and that the gains were greater when

<div align="center">TABLE I</div>

Influence of Supplementary Casein, Cystine, and Methionine on Liver Lipid
Content of Adult Male Rats

The roman numerals in the first column refer to the series. The cystine and methionine contents of the diets in the individual series are (mg. per cent) respectively as follows: Series I (17 and 155), Series II (217 and 620), Series III (417 and 620), Series IV (817 and 620), Series V (617 and 775). The cystine and methionine in the casein supplements were calculated from the analysis given by Kassell and Brand (13). The duration of the experiments was 21 days. The data in the last column were obtained in a previous publication (8)   Liver lipids were calculated on the fresh basis. The values in parentheses are the variations for the individual rats. Eight rats were employed in Series I, and seven in each of the remaining series.

Group and series No	Supplements added to 100 gm diet			Average daily food intake	Change in weight	Liver lipids (adult rats)	Liver lipids (young rats (8))
	Casein	Cystine	Methionine				
	gm	mg	mg	gm	per cent	per cent	per cent
8-I	0	0	0	11.8	−1.1	24 2 (10.5–34.2)	23.6 (15.9–34.4)
3-II	0	200	465	10.8	+9.3	9.5 (7.4–12.0)	11.2 (7.0–15.0)
17-II	15	149	0	10.8	+21.3	9.9 (7.4–12.8)	26.3 (22.2–32.4)
4-III	0	400	465	11.3	+10.5	10.5 (7.3–13.6)	14.0 (7.2–18.5)
18-III	15	349	0	10.8	+22.4	10.5 (6.2–16.8)	20.6 (13 0–28.4)
6-IV	0	800	465	10.0	+10.3	9.5 (8.2–11.6)	15.7 (11.6–20.3)
19-IV	15	749	0	9.9	+21.7	10.8 (7.8–14.7)	26.5 (21.6–33.0)
12-V	0	600	620	11.3	+7.1	11.7 (9.0–15.6)	9.9 (5.8–13.0)
15-V	20	532	0	11.0	+21.2	11.1 (7.1–17.9)	19.8 (14.6–30 4)

casein was used as the supplement.   While no data were obtained on the total lipid content of the whole animals, it was strikingly evident from mere inspection that in all of these older animals large amounts of fat were deposited.   This was not the case in the younger rats.   In view of these observations and the reports from Mitchell's laboratory (9–11), it is our opinion that less methionine is needed for increases in weight in the older rats and hence more is thus available for lipotropic action.   This will serve to explain the differences between the results obtained with the adult animals and our previous findings (8) with young rats.   In Series II to IV inclusive the content of dietary amino acids other than methionine and

cystine was increased 4-fold when casein was used as the supplement.   In Series V this increase was 5-fold.   In spite of these wide variations, the liver lipid content of all of the older rats was essentially the same.   Certainly in these adult animals the lipotropic action can be ascribed to the methionine content of the diets only.

SUMMARY

In experiments with adult male rats (weight 250 gm.) a basal diet containing 5 per cent casein was, so far as lipotropic action is concerned, just as effective when supplemented with methionine and cystine as when supplemented with sufficient amounts of cystine and casein to equalize the content of the two sulfur-containing amino acids in the two diets.   When the basal diet was supplemented with casein, the protein content was increased from 5 to 20 per cent in some instances and from 5 to 25 per cent in others. Evidently the 4- and 5-fold increases in dietary amino acids other than cystine and methionine were without influence on liver lipid content and it is concluded that in these adult rats the effects of the diets on liver lipid content may be explained entirely by their content of cystine and methionine.

BIBLIOGRAPHY

1 Best, C H , and Lucas, C C , Vitamins and hormones, New York, 1, 1 (1943).
2. McHenry, E. W , and Patterson, J M., *Physiol. Rev* , 24, 128 (1944).
3. Channon, H J , Manifold, M. C , and Platt, A. P , *Biochem. J.*, 34, 866 (1940).
4. Tucker, H. F., Treadwell, C R , and Eckstein, H C , *J. Biol Chem* , 135, 85 (1940).
5. Tucker, H. F., and Eckstein, H. C , *J Biol Chem.*, 126, 117 (1938).
6. Beeston, A. W., and Platt, A. P , *Chem and Ind* , 17, 557 (1939).
7. Channon, H. J , Mills, G. T., and Platt, A. P., *Biochem. J.*, 37, 483 (1943).
8 Treadwell, C. R., Groothuis, M , and Eckstein, H C , *J. Biol. Chem.*, 142, 653 (1942).
9. Hamilton, T. S., *J. Nutr.*, 17, 565 (1939).
10. Smuts, D. B., *J Nutr* , 9, 103 (1935)
11 Mitchell, H. H , and Carman, G G , *Am J Physiol.*, 74, 398 (1926)
12. Osborne, T. B., and Mendel, L B , *J Biol Chem* , 37, 572 (1919)
13. Kassell, B , and Brand, E , *J Biol Chem.*, 125, 435 (1938).
14. Tucker, H F , and Eckstein, H. C , *J Biol Chem* , 121, 479 (1937)
15. Graeser, J. B , Ginsberg, J E , and Friedemann, T E., *J. Biol. Chem* , 104, 149 (1934).

ENZYMATIC SYNTHESIS OF ACETYL PHOSPHATE*

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Earlier reported preliminary results with bacterial enzyme preparations (1, 2) led to a formulation of pyruvate oxidation to yield acetyl phosphate as the primary reaction product in the following manner.[1]

$$CH_3CO \cdot COOH + H \cdot OPO_3H_2 \xrightarrow{\quad -2H \quad} CH_3CO \cdot OPO_3H_2 + CO_2$$
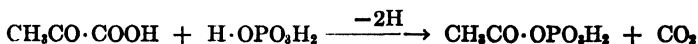
In order to prove this suggestion, a study of the chemistry of acetyl phosphate was undertaken and eventually a method of determination and a simple synthesis were worked out (3). With these available, on oxidation of pyruvate the appearance of a labile organic phosphate was observed (4) which closely resembled acetyl phosphate. The present communication reports upon the isolation of this intermediary and its identification with acetyl phosphate. The second part of the paper deals with the linking between pyruvate oxidation and adenylic acid phosphorylation through acetyl phosphate as the intermediary. This transfer of the phosphoryl group between acetic and adenylic acid was found to be reversible.

Enzyme Preparations

Bacillus acidificans longissimus Lafar was used as the source of the enzyme system. This organism is classified ordinarily with the *delbrueckii* group (5). The original culture had been obtained in 1937 from the Institut für Gaerungsgewerbe in Berlin. This culture had been discontinued, and, when these studies were taken up again, such strains of *Lactobacillus delbrueckii* as were available in this country were tried. Three strains were kindly supplied by Professor W. H. Peterson. None of them was found to contain the stable pyruvic acid enzyme, although at least one, *Lactobacillus delbrueckii* 3, of this collection was almost indistinguishable in shape and behavior from *Bacillus acidificans*. Eventually, a culture of the original *Bacillus acidificans longissimus* was secured from England through the courtesy of Dr. J. G. Davis who fortunately had continued the cultivation of the organism in which he had discovered (6) the unusually stable pyruvic oxidase system here under investigation.

[1] To avoid a discussion of the state of ionization, seemingly not necessary here, throughout this paper compounds are written in the form of free acids.

The organism was grown in a medium containing 15 gm. of malt extract (Difco), 20 gm. of glucose, 10 gm. of calcium carbonate, and 70 ml. of yeast autolysate in 1 liter of tap water. After 25 hours incubation at 37°, the remaining calcium carbonate was removed by low speed centrifugation. The bacteria were collected by continuous centrifugation through a cream separator (McCormick-Deering). The paste was washed once by suspending it in one-tenth the original volume of a solution containing 0.6 per cent sodium chloride and 0.2 per cent sodium bicarbonate.

Drying Procedure—The washed paste is spread about 1 mm. thick on large watch-glasses. It is dried quickly in vacuum desiccators over phosphorus pentoxide. When samples of 1 to 2 gm. of the dry material are handled, most of the water may be removed in $\frac{1}{2}$ to 1 hour, if a good vacuum, preferably by means of an oil pump, is applied. To complete the desiccation the material is kept *in vacuo* overnight. 1 to 1.5 gm. of dry material was obtained per liter of culture fluid.

Characteristics of Dry Material—In the early stages of these studies an acetone preparation had been used (7). Vacuum drying yields preparations of greater stability and more varied activity. A further advantage is that these preparations contain the pyruvic oxidation system in extractable form. All experiments reported in this communication were carried out with vacuum-dried bacteria or derivatives thereof.[2]

The stability of these preparations is remarkable. The majority of the experiments reported here were done with preparations kept since 1938. First they were kept for 3 years at refrigerator temperature. Then, as a slight decrease of activity was observed, the storage was continued at −35°. No further loss of activity has occurred under these conditions. The activity of dry preparations from different batches was rather constant. The Q_{O_2} with pyruvate was 7 to 10, at pH 6.3 and 37°.

Preparation of Enzyme Extracts—The dry material is very carefully ground to a dust-fine powder and suspended in 10 to 15 times its weight of 0.03 M secondary phosphate solution. Vigorous shaking is needed to extract the enzyme. The suspension is shaken for 30 minutes at 37° with 160 excursions per minute. A slightly misty, yellow-brown solution is obtained by the use of an ordinary centrifuge. Without loss of activity, perfectly clear solutions were obtained by centrifuging at 15,000 R.P.M. The protein content of such extracts is approximately 1 per cent. The extractability of

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[2] These preparations contain the glycolytic system. The fermentation is accompanied by an extensive disappearance of inorganic phosphate. In a typical experiment, 85 mg of dry bacteria were suspended in 1 ml. of 0 1 M bicarbonate solution containing 1 13 mg of phosphate P and 50 mg of glucose. Acid formation was measured manometrically with pure carbon dioxide as the gas phase In 45 minutes 917 microliters of lactic acid were formed and 0.64 mg. of inorganic P had disappeared.

the enzyme system varied from sample to sample, sometimes being lower than in the cited experiments.

Example—In a 100 ml. Erlenmeyer flask 0.55 gm. of powdered dry bacteria, 2.5 ml. of 0.1 M disodium hydrogen phosphate, and 4.5 ml. of water were shaken for 30 minutes in a bath at 37.5°. 4 ml. of extract of pH 6.5 were obtained. To 1 ml. of enzyme solution 0.25 ml. of molar pyruvate was added, and oxygen consumption was measured at 37.5°. 695 microliters of O_2 were used in 60 minutes. This corresponds to a Q_{O_2} of 70.

In a few experiments enzyme solutions derived from *Clostridium butylicum* were used. This material was kindly supplied to us by Dr. H. J. Koepsell and Dr. Marvin J. Johnson. It was the dry material obtained by lyophilizing the extract of frozen organisms. The procedure is described in the paper of Koepsell and Johnson (8) on pyruvate metabolism in extracts of *Clostridium butylicum*.

Analytical Procedures

Manometric measurements were carried out in the usual manner with conical and oblong rectangular vessels of 15 to 20 ml. volume. The oblong type (no inner cup) was used when efficient shaking of a heavy, rapidly metabolizing suspension was required. The metabolism of pyruvate was followed either by measuring oxygen consumption, with alkali in the inner cup, or by following CO_2 production. With large amounts of pyruvate the respiratory quotient is very exactly 2 and accurate calculation of the total CO_2 is possible by the use of the following formula, slightly modified from that of Warburg (9).

$$\text{Microliters } CO_2 = \text{mm. reading} \times \frac{K_{CO_2} \times K_{O_2}}{K_{O_2} - \dfrac{K_{CO_2}}{\text{R.Q.}}}$$

A close agreement was found between manometric and titrimetric estimation of pyruvate utilization (*cf.* Tables II and V and Table I with Fig. 1).

Pyruvate was determined titrimetrically by the iodometric method of Clift and Cook (10).

Phosphate Fractions—Inorganic phosphate, referred to as P_i, was estimated colorimetrically according to Fiske and Subbarow (11). The color was equalized by warming to 37° for 7 minutes (12).

Acetyl phosphate, referred to as P_{ac}, was determined by the procedure of Lipmann and Tuttle (3) as the difference between apparent phosphate and the phosphate precipitable with alcoholic $CaCl_2$ at neutral reaction.

Adenyl polyphosphate, is referred to as P_7, representing the "easily hydrolyzable" phosphate liberated by heating with normal hydrochloric acid for 7 minutes in a boiling water bath.

Acetic acid was determined by steam distillation. 100 ml. of distillate were collected and titrated with N/60 sodium hydroxide. A standard curve was prepared. It deviated slightly from theory only for titrations requiring below 1 ml. of hydroxide solution.

Silver was titrated in strongly acid solution with 0.01 N sulfocyanide, a drop of 20 per cent ferric ammonium sulfate being used as indicator.

Preparations. Sodium Pyruvate—A 15 to 30 per cent solution of freshly distilled pyruvic acid is cooled and neutralized slowly by adding solid sodium bicarbonate, the amount used being not more than 90 per cent of the bicarbonate needed for complete neutralization. The sodium pyruvate is precipitated by mixing with 5 to 6 volumes of acetone and keeping it in the refrigerator overnight. A 95 per cent pure preparation was obtained in good yield without reprecipitation.

Acetyl phosphate was synthesized according to Lipmann and Tuttle (3). The silver salt was stirred with a 10 per cent excess of sodium chloride. The filtered solution was kept frozen at $-35°$. In this form the solution may be kept for weeks without loss.

Adenylic acid was a preparation which had been kindly supplied to us by Dr. P. Ostern.

Adenyl pyrophosphate was prepared by the procedure described by Dorothy M. Needham (13).

Interdependence of Phosphorylation and Pyruvate Utilization

In preparation for an isolation of the labile phosphate compound, the conditions of its formation and stabilization were studied, especially to find the optimum concentration of the reactants, pyruvate and phosphate. In Table I the yield of acetyl phosphate is compared with the initial phosphate concentration. The tabulated end-point analyses are supplemented by the curves of Fig. 1, showing the time course of oxidative decarboxylation. It appears that, except for the lowest concentration of phosphate, at first the carbon dioxide output is relatively independent of phosphate concentration. The fairly sudden drop in carbon dioxide formation which occurs at lower phosphate concentrations is due to a depletion of inorganic phosphate. It may be concluded from these data that the system is saturated with phosphate at concentrations of 5 to 6 mM per liter. Conversion of inorganic phosphate into acetyl phosphate leads, at low phosphate concentration, to exhaustion of the supply of inorganic phosphate and consequently to the standstill of the reaction. At higher phosphate concentration continuous decomposition of accumulating acetyl phosphate, spontaneous and enzymatic, yields enough inorganic phosphate to metabolize the total pyruvate at a fairly homogeneous rate (*cf.* the last two lines in Table I).

TABLE I

Ratio of Acetyl Phosphate Formation to Pyruvate Utilization

Suspension of 40 mg. of dry bacteria in 0.013 M sodium acetate and 0.033 M sodium fluoride solution, with addition of varying amounts of inorganic phosphate.   Total volume per vessel 0.75 ml., bath temperature 37°, air as gas phase.   48 micromoles of pyruvate added at the start.

P_i, initial	Incubation time	P_t	P_{ac}	Pyruvate (titrimetric) disappearance	$\dfrac{P_{ac}}{\text{Pyruvate}/2}$
	min	*micromoles*	*micromoles*	*micromoles*	
2.0	40	0.8	1.2	1	
6.5	40	0.8	4.8	12.5	0.77
16.5	40	5.2	11.3	30	0.75
16.5	100	3.7	12.8	44.4	0.57
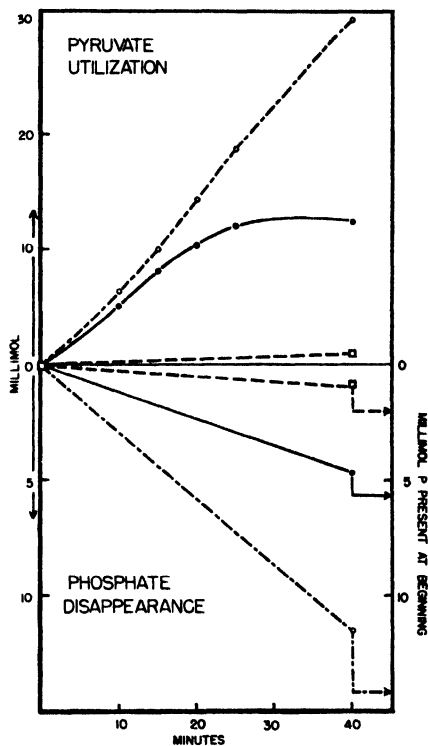
FIG 1. The effect of phosphate concentration on initial rate and time course of pyruvate oxidation.   The rising curves represent carbon dioxide evolution, the corresponding falling curves acetyl phosphate formation.

As a convenient expression for the ratio between oxidative decarboxylation and phosphorylation the quotient P_{ac}/O_2, equaling $P_{ac}/(\text{pyruvate}/2)$, was used. The values of this phosphorylation quotient, listed in the last columns of Tables I and II, serve as a measure of the stabilization of acetyl phosphate. The stabilizing action of fluoride is pronounced. By 0.03 M fluoride the breakdown of added acetyl phosphate was usually inhibited somewhat more than 50 per cent. An acyl phosphate-splitting enzyme has been found quite commonly in bacterial and animal cells.

TABLE II

Effect of Fluoride on Stabilization of Acetyl Phosphate

Experiment No	Character of preparation	Fluoride	Initial pyruvate	$-O_2^*$	P_{ac}	P_i	$\dfrac{P_{ac}}{O_2}$
		mole per l.	*micromoles*	*micromoles*	*micromoles*	*micromoles*	
1	Extract, 65 min incubation	0	200	27.5	16	24	0.58
		0 033	200	28	20.5	21	0.73
		0.033	0	4	0.5	41	
2	Suspension, 42 mg per vessel, 140 min incubation	0	72	35†	5	20	0.14
		0 033	72	34 5	19	8.4	0.55
3	Suspension, 50 mg per vessel, Preparation 38	0.037	250	27.5	8.2	24.3	0.3
	Preparation 39, 80 min incubation	0.037	250	29.5	19.3	13.2	0.65

* In Experiment 1 oxygen consumption was measured, while in Experiments 2 and 3 carbon dioxide production was measured and oxygen consumption was calculated for R Q. 2.

† In accordance with the manometric measurement only traces of pyruvate were found chemically at the end of this experiment.

The values for the phosphorylation quotient are seen more or less to approach unity. *1* mole of acetyl phosphate may form, therefore, per mole of oxygen used and per oxidation of a total of *2* moles of pyruvate. It had been shown previously (7) that half of the pyruvate is oxidized non-enzymatically by hydrogen peroxide arising in the primary enzyme reaction through reoxidation of the leucoflavin enzyme.

Enzymatic—$CH_3COCOOH + H_3PO_4 + \text{flavin-thiamine enzyme} \rightarrow$

$$CH_3COOPO_3H_2 + CO_2 + \textit{leuco}\text{flavin-thiamine enzyme}$$

$$\textit{Leuco}\text{flavin} + O_2 \rightarrow \text{flavin} + H_2O_2$$

Non-Enzymatic—$H_2O_2 + CH_3COCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2O$
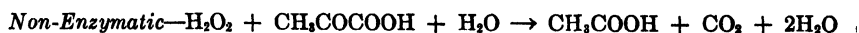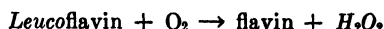
The secondary oxidation of pyruvate with hydrogen peroxide was found not to yield acetyl phosphate, at least not in water or at low phosphate concentrations. However, Baer (14) described recently a non-enzymatic oxidation by lead tetraacetate of pyruvic and phosphoric acids which yields acetylphosphoric acid.

Isolation of Acetyl Phosphate

Outline—1 gm. samples of dry bacteria were shaken, in the presence of fluoride, with phosphate and pyruvate in a proportion of 1:2.5, until the pyruvate had nearly disappeared. Then 5 times the volume of ethanol was added. After centrifugation, the supernatant fluid was reduced *in vacuo* to a small volume. From this, acetyl phosphate was precipitated with concentrated silver nitrate solution and ethanol. The crude silver fractions from several such experiments were combined. For final purification the silver fractions were dissolved with equivalent amounts of sodium chloride and reprecipitated by excess silver nitrate. After several such reprecipitations pure silver acetyl phosphate was obtained.

Enzymatic Synthesis. Deproteinization

1 gm. of dry bacteria was finely powdered and rubbed with 10 ml. of a solution, 0.05 M in potassium phosphate, of pH 7, and 0.05 M in sodium fluoride. The pH of the suspension was 6.3. A total of 2.5 ml. of 0.5 M sodium pyruvate solution was added to the 10 ml. The suspension was shaken with air at a rate of 150 oscillations per minute in a bath of 37°. To follow the reaction, 1 ml. of the suspension was brought into a manometric vessel. After 2 hours the rate of reaction had slowed down considerably. At that time 113 micromoles of CO_2 had been evolved from 125 micromoles of pyruvate. Usually 60 to 70 per cent of initial inorganic phosphorus had then been converted to acetyl P.

The experiment was interrupted now, and the bacterial débris was removed by centrifugation and washed with 5 ml. of water. . The combined centrifugates, amounting to about 15 ml., were cooled, and with a few drops of normal nitric acid the pH was adjusted to approximately 4. Without delay 75 ml. of ethanol were added, the mixture was kept in ice for 30 minutes to promote flocculation, and the precipitate was centrifuged off. The acidification makes protein precipitation with ethanol rather complete. Furthermore, subsequent vacuum distillation removes from the acid fluid most of the free acetic acid, which interferes with purification of the silver salt of acetyl phosphate. Acetyl phosphate is sufficiently stable at pH 4 (3), especially in alcoholic solution.

Crude Silver Fraction—The slightly turbid alcoholic fluid was brought into a 200 ml. Claisen flask. The alcohol and most of the water were then dis-

tilled off *in vacuo*, with an outside temperature of 30–32°. The receiver contained 1 ml. of normal sodium hydroxide and was packed in ice. After 30 minutes, when the fluid had been boiled down to about 2 ml., the distillation was discontinued. The flask was cooled in ice and left upright for a few minutes to collect the fluid in the bottom. The fluid was transferred with a finely drawn out pipette, the flask being washed twice with a few drops of water. A little flocculent precipitate was centrifuged off. An amount of 2.5 ml. of clear, slightly greenish fluid was obtained containing 6.6 mg. of acetyl P and only traces of inorganic phosphorus, with reference to the initial 15 mg. of inorganic P a yield of 45 per cent. The absence of inorganic phosphate is due to its precipitation as alkali phosphate from the alcoholic solution. Without detectable loss, such solutions could be kept frozen for a week or longer.

A crude silver precipitate was now obtained in the following manner. The 2.5 ml. remaining after vacuum distillation had been kept frozen at −35° for 3 days. To the frozen solution of pH 5.5 were added 1.6 ml. of 20 per cent silver nitrate solution and 4 ml. of ethanol. A bulky precipitate formed, and the mixture was kept for 3 hours at −35°. With addition of an equal volume of alcohol, the ethanol concentration was brought to 66 per cent, and the white precipitate centrifuged off and washed dry with alcohol and ether. 152 mg. of a slightly brownish product were obtained which contained 2 8 per cent of acetyl P, traces of inorganic phosphate, and 46 per cent of silver. Disilver monoacetyl phosphate contains 8.8 per cent of P and 61 per cent of silver. The first precipitate, therefore, contained approximately 30 per cent acetyl phosphate.

In the form of the silver salt even the impure product may be kept practically indefinitely. Moisture and light, of course, have to be excluded, and it is preferable to keep the desiccator in the cold room.

Purification of Silver Fraction

From four analogous experiments, nearly 0.5 gm. of crude silver salt of 27.5 per cent purity was obtained. Of this, 457 mg., containing 1.96 mM of silver, were suspended in 7 ml. of ice-cold water, and stirred mechanically with outside cooling until a homogeneous suspension resulted. Then, 1.8 ml. of molar sodium chloride were added dropwise, and the stirring was continued for 15 minutes. A dirty white precipitate separated out on standing. Without removal of the precipitate, 0.5 ml. of molar calcium nitrate solution was added, followed by 1 ml. of ethanol. Only a slight precipitate of calcium phosphate formed. After a few minutes standing, the combined precipitates were centrifuged off. All manipulations described in this and the following paragraphs require, of course, effective cooling, although it was not found necessary to use a cooled centrifuge.

To the clear and colorless supernatant 3 ml. of 20 per cent silver nitrate solution were added, and a rather copious white precipitate settled out. The solution was frozen briefly and while frozen its centrifugation was started. On centrifugation the solution had thawed and the precipitate (I) had collected at the bottom. To the supernatant an equal volume of ethanol was added and it gave an appreciable further precipitation (II). Precipitate II was dried with alcohol and ether.

Precipitate I was dissolved, without washing, by excess nitric acid. The solution was then neutralized cautiously to pH 5 with sodium hydroxide solution. An equal volume of ethanol was added and the slightly brownish (silver oxide) precipitate was dried with alcohol and ether. Precipitates I and II, each amounting to somewhat over 30 mg., gave very similar analyses and were combined.

This product contained 6.7 per cent acetyl P, 1.5 per cent of inorganic phosphorus, and 43 per cent of silver. The purity had increased in this step to around 65 per cent. Some decomposition, however, had occurred as indicated by the appearance of inorganic phosphate. This was due mostly to dissolving in acid and reprecipitation with alkali, a procedure which is effective with barium fractions but not recommendable with silver preparations.

The 65 mg. of the combined preparations, containing 0.26 mM of Ag, were suspended in 1 ml. of water. When the material was stirred, the reaction of the suspension became faintly acid to Congo red paper, while much of the compound dissolved. With continued stirring 0.23 ml. of normal sodium chloride was added, also 3 drops of ethanol to counteract frothing. The silver chloride was filtered off by suction on a micro funnel (15) and rinsed with 0.25 ml. of water. The filtrate was neutralized with 3 drops of 0.35 N barium hydroxide solution, and a drop of normal calcium nitrate and 0.25 ml. of ethanol were added. The calcium phosphate was filtered off and 0.7 ml. of 20 per cent silver nitrate solution was added. After the mixture had stood in ice water for 20 minutes, the silver precipitate was collected on the centrifuge, this time without addition of more alcohol. It was washed with 50 per cent alcohol and dried with alcohol and ether.

The yield was 23 mg. The analytical data, given on the third line in Table III, indicate the rather high degree of purity. After analysis there remained 12 mg. They were suspended in 1.25 ml. of water. Most went into solution, leaving some flocculent yellowish material undissolved. The pH of the solution was about 6, and, in order to neutralize fully, a little freshly precipitated silver carbonate was added. The solution was sucked through a micro filter and reduced in vacuo until crystals started to settle out. An equal volume of alcohol was added now, and the partly crystalline material was collected on the centrifuge, washed with alcohol and ether, and

kept overnight in a vacuum desiccator over phosphorus pentoxide. The yield was 8.1 mg. The analysis of this compound shown on the fourth line in Table III agrees with the composition of disilver monoacetyl phosphate.

Since the acidified solution of the compound decomposes readily on heating (3), the acetic acid content is easily determined by steam distillation. The identity of the volatile acid was ascertained in the following manner. The steam distillate was neutralized with some excess of sodium hydroxide, dried on the steam bath, and a Duclaux distillation was carried out. In practical agreement with a yield of 43.3 per cent for pure acetic acid, 42.4 per cent of the volatile acid present in the isolated silver salt distilled with the first 10 ml. in our apparatus. For further identification, a little freshly prepared uranyl carbonate was dissolved with cautious heating in a drop of distillate. On addition of a crystal of sodium chloride typical pyramids of sodium uranyl acetate formed copiously.[3]

TABLE III

Progress of Purification of Silver Salt

The values are given in per cent.

Fractions	P_i	P_{ac}	Ag	Acetic acid
Initial..	0.5	3.0	46	
1st reprecipitation	1 9	6.2	43	
2nd "	0	7.3	52	17.2
3rd "	0	8.5	63	17.4
Calculated for $C_2H_3O_5PAg_2$. .		8.78	61.0	17.0

Coupling between Pyruvate Oxidation and Adenylic Acid Phosphorylation

Rather crude experiments had indicated earlier (1) that an enzymatic transfer of phosphoryl may occur from acetyl phosphate to adenylic acid. These observations have been corroborated now. It appears that in the bacterial enzyme system the over-all transfer of inorganic phosphate to adenylic acid by way of pyruvate oxidation can be differentiated into two phases, the oxidative generation of the energy-rich phosphate bond in acetyl phosphate and the transference of phosphoryl from acetyl phosphate to adenylic acid   The analysis of the first step of this sequence was described in the preceding paragraphs. The following will deal with the coupling reaction and the distinction of its two phases, generation and transfer of bonded phosphate.

Yield of Adenyl Phosphate—The current nomenclature, referring the series of adenyl phosphates to adenosine as mother substance, is misleading

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[3] For carrying out these identity tests, I am thoroughly indebted to Dr. H. Albert Barker, Berkeley, who at the time of these experiments was a guest of this laboratory.

because this usage treats as equal two essentially different phosphate bond types. Adenylic acid, the adenosine *mono*phosphate, contains an ordinary ester bond only, while adenosine *di*- and *tri*phosphate contain *one* and *two* energy-rich bonds in addition to the ester bond. We use a nomenclature referring to adenylic acid as the mother substance of the series. This avoids ambiguity and focuses attention on the metabolically active phosphoryl radicals. As a group term adenyl phosphate is used. Adenyl di- or pyrophosphate, and adenyl monophosphate are conveniently shortened to ad$\sim$ph$\sim$ph and ad$\sim$ph respectively. The sign $\sim$ph is used for the energy-rich phosphate bond in its various forms, as has been recommended earlier (16).

As shown in Table IV, between 0.25 and just over 0.5 mole of P_7 per mole of oxygen appeared. There is significant variation with different batches

TABLE IV

Ratio of Oxygen Uptake to Adenylic Acid Phosphorylation

Preparation No.	Initial adenylic acid	P_7	Oxygen consumed	$\dfrac{P_7}{O_2}$	Outline of experiment
	micromoles	*micromoles*	*micromoles*		
1	26	4 1	17.0	0.24	Suspension of 36 mg. dry bacteria
5	26	7.7	16.0	0 48	in 0 04 M NaF-phosphate solu-
2.3	26	9 6	18.2	0.53	tion; 100 micromoles pyruvate added, 60 min. incubation at 37°
1	27	3.1	13.7	0.23	Same as above, 50 min. incubation
3.4	40	0	12.5	0	0 5 ml phosphate extract from 40 mg. bacteria, 60 min. incubation

of bacteria due presumably to a variation in enzyme distribution and similar to that found for acetyl phosphate accumulation (Table II). Fluoride stabilizes the easily hydrolyzable phosphate. Breakdown of added adenyl diphosphate was variable but pronounced, although in the presence of fluoride it was only 10 to 13 per cent per hour.

A yield of adenyl phosphate lower than of acetyl phosphate (Table II) may indicate either a sluggishness of transfer or an equilibrium between the two compounds. Some clarification was brought through simultaneous estimation of the two phosphate fractions. In the experiment shown in Table V the reaction was interrupted when, mole per mole, pyruvate breakdown amounted to slightly more than twice the inorganic phosphate present initially. At that time the rate of reaction just started to slow down, and inorganic phosphate had disappeared almost completely. In the absence

of adenylic acid, acetyl phosphate was the only product of phosphorylation. With adenylic acid present, however, the phosphoryl is distributed between adenyl phosphate and acetyl phosphate. Obviously the phosphorylation of adenylic acid is branching off from the oxidative reaction of which it is not an integral part. Adenylic acid even slightly depressed the rate of pyruvate oxidation.

TABLE V

Ratio of Oxygen Uptake to Total Phosphate Disappeared

39 mg. of bacteria per vessel were suspended in 0.85 ml. of 0.03 M NaF solution containing 22.2 micromoles of inorganic P. 73 micromoles of pyruvate were added at the start   37°, air, 105 minutes incubation.

Preparation No	Adenylic acid added	Pyruvate (titrimetric)	CO_2 (mano-metric)	P distribution			
				P_i	$-\dfrac{P_i}{O_2^*}$	P_7	P_{ac}
	micromoles	*micromoles*	*micromoles*	*micromoles*		*micromoles*	*micromoles*
7	11.2	−54.6	52	−20.3	0.75	3.7	15.3
7	0	−60.5	63	−21.6	0.72	0.8	19.8
2.3	11.2	−51.0	53	−21.1	0.83	10.6	9.3
2.3	0	−63.5	70	−18.0	0.57	1.6	15.8

* O_2 = pyruvate/2.

TABLE VI

Resolution of Coupling Reaction into Oxidative and Anaerobic Stages

Suspension of 45 mg. of dry Preparation 2.3 in 1.0 ml. of 0.025 M NaF solution incubated at 37°. All data are given in micromoles.

Procedure	Adenylic acid added	Pyruvate	Phosphate distribution		
			P_7	P_{ac}	P_i
Stage 1. Incubation in oxygen, at start	0	75.5	0.5	0	20.4
Same after 115 min		2.3	1.3	14.4	5.8
Stage 2. 60 min. incubation in nitrogen	11.2		6.5	5.2	9.7
	0		1 3	6.9	13.0

Resolution of Coupling into Aerobic and Anaerobic Phase—The following experiments show directly the two phase nature of the over-all coupling. In the experiment of Table VI, pyruvate was oxidized and acetyl phosphate accumulated to the extent shown by the analysis at the end of the aerobic period. Air was replaced then by nitrogen in order to exclude any further energy supply and adenylic acid was added from the annex of the vessel. Incubation was continued for 1 more hour. The result of the anaerobic period is a migration of about half of the acetyl-bound phosphorus to ade-

nylic acid, the phosphoryl distribution being now 55 per cent bound to adenylic acid and 44 per cent bound to acetyl. The experiment furthermore exemplifies the unavoidable loss of acetyl phosphate, largely by enzyme action, amounting in this case to 50 per cent per hour.

The equivalence between enzymatic and synthetic acetyl phosphate is emphasized by the experiment of Table VII. In this experiment a solution of acetyl phosphate was used which was derived from pure crystalline silver monoacetyl phosphate. The enzyme preparation was the same as in the experiment of Table VI and practically the same amount of acetyl phosphate was present at the start of anaerobic incubation. The adenylic acid concentration, however, was 50 per cent higher here. Accordingly more phosphoryl shifted to the adenylic acid side than in the earlier experiment. The phosphoryl distribution after incubation was 73 per cent adenyl-bound and 27 per cent acetyl-bound.

TABLE VII

Transfer of Acetyl-Bound Phosphorus to Adenylic Acid

The same preparation and the same proportions as in the experiment of Table VI. All data are given in micromoles. Here, however, 15 micromoles of synthetic acetyl phosphate are added and incubation is in nitrogen only.

Incubation time	Adenylic acid added	P_7	Per cent of $(P_7 + P_{ac})$	P_{ac}	Per cent of $(P_7 + P_{ac})$	P_t
At start...		2.1		15.0		4.0
After 60 min.	15.6	8.0	73	2.9	27	10.0
" 60 "	0	2.1		6.9		12.1

Phosphorylation of Fatty Acid Carboxyl by Adenyl Pyrophosphate

The data of the experiments recorded in the Tables V to VII suggested that the transfer of phosphoryl from acetyl phosphate to adenylic acid approached a state of equilibrium. They did not, however, exclude rigidly the possibility that the incompleteness of reaction was due to sluggishness. To prove the establishment of a true equilibrium, the reverse reaction had to be demonstrated. In the experiments of Table VIII adenyl pyrophosphate and acetate, or butyrate, were incubated with enzyme preparations from *Bacterium delbrueckii* and *Clostridium butylicum*. Butyrate was included because Koepsell and Johnson had privately communicated to us observations indicating in *Clostridium butylicum* the formation of some butyryl phosphate as well as acetyl phosphate. Small amounts of labile phosphate appeared on incubation with both fatty acids. In the case of the *Clostridium butylicum* enzyme a homogeneous solution was used. *Bacterium delbrueckii* had to be used in a suspension because our extracts did not contain the transfer enzyme (*cf.* Table IV). Although the results were more uni-

form with the soluble enzyme, phosphate transfer from adenyl to acyl was evident with both enzymes.

It is probable that in these experiments a final equilibrium was not yet obtained from either side. Nevertheless, the data may serve for an approximate calculation of the equilibrium constant of the reaction

$$\text{Adenyl}\sim\text{ph} + \text{acetyl}\sim\text{ph} \rightleftharpoons \text{adenyl}\sim\text{ph}\sim\text{ph} + \text{acetate}$$
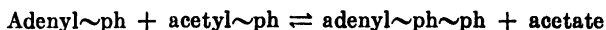
In this equation adenyl monophosphate is assumed the primary reactant. Meyerhof *et al.* (17) showed for the similar coupling between glyceraldehyde phosphate oxidation and adenylic phosphorylation that not adenylic acid but the adenyl phosphate, containing already one energy-rich phosphate group, was the true acceptor. When adenylic acid is active,

<div align="center">TABLE VIII</div>

Phosphorylation of Fatty Acids by Adenyl Pyrophosphate

Enzyme preparation	Fluoride	Before incubation			After 50 min incubation, 37°		
		Acetate	Butyrate	P_7 added	P_7	P_{ao}	P_i
	mole per l	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*micromoles*
Suspension of dried *B.*	0.03	0		0	0.2	0.2	4.5
delbrueckii, No 2 3;		200		5.8	5.2	1 2	6.2
total volume 0 58 ml.	0.03	130		28.5	23 0	1.2	8.9
Extract of frozen *Cl.*	0.02	100		18.0	15.0	1 0	9.6
butylicum (8), total			70	18.0	11 0	2 0	9 6
volume 0 55 ml				18 0	16.6	0 4	8.6
	0.008	100		18 0	14.2	1 8	9.6
			70	18 0	14.2	2 4	9.0
				18 0	16 0	0 6	9.0
				0	0.2	0	7.6

as it is in our preparations, most probably a phosphodismutation with some adenyl diphosphate present yields adenyl monophosphate in the manner described by Kalckar (18). For this reason the data of Table VIII, obtained for the reverse reaction, right to left, are considered more suited for a calculation because there the primary reactants were added to the system.

For the estimation of the constant,

$$K = \frac{\text{ad}\sim\text{ph}\sim\text{ph} \times \text{acetate}}{\text{ad}\sim\text{ph} \times \text{ac}\sim\text{ph}}$$

the concentration of adenyl monophosphate (ad$\sim$ph) was roughly calculated (*cf.* (18)) as half the difference between added and remaining easily

hydrolyzable phosphate. The molar concentration of ad~ph~ph is obtained by halving P_7 after deduction of ad~ph. With the data of the *Clostridium* experiment, K for acetate is

$$\frac{\dfrac{14\,2 - 1.9}{2} \times (100 - 1.8)}{1.8 \times 1.9} = 177$$

The constant for butyrate would be somewhat lower.

Assuming the value of 177 for the constant as an acceptable approximation, we may proceed to calculate the difference between the bond energies in acetyl and adenyl phosphate respectively. Using the equation (19),

$$\Delta F_0 = -RT \ln K = -4.58 \times T \times \log K$$

we obtain for 37°

$$\Delta F_0 = -4.58 \times 310 \times 2.5 = -3.55 \text{ kilocalories}$$

The bond energy of the carboxyl phosphate is therefore some 3 kilocalories higher than the average energy of 12 kilocalories of the energy-rich bond. It will amount to around 15 kilocalories.

Acetyl Phosphate Formation with Other Organisms

Koepsell and Johnson (8) found recently with extracts of *Clostridium butylicum* anaerobic splitting of pyruvate to acetyl phosphate (private communication), hydrogen, and carbon dioxide, and Utter and Werkman (20) demonstrated with *Escherichia coli* extracts a decomposition of pyruvate to acetyl phosphate and formate.

$$CH_3COOPO_3H_2 + H_2 + CO_2 \qquad (a)$$

$$CH_3CO \cdot COOH + H \cdot OPO_3H_2$$

$$CH_3COOPO_3H_2 + HCOOH \qquad (b)$$
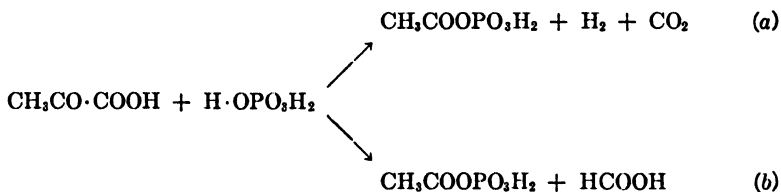
Formation of the energy-rich acyl phosphate bond appears here as the result of intramolecular rearrangement in the phosphate-pyruvate addition product. The rearranged molecule eventually splits into two or more parts, depending on the particular enzyme or enzyme system. The probability of such a course of reaction is emphasized by indications for the reversibility of the "phosphoroclastic" splitting of pyruvate. Such indications are found in the inhibition of reaction (a) by hydrogen (21), and in unpublished experiments of ours on the reversal of reaction (b). The formation of small amounts of keto acid was observed on incubation of *Escherichia coli*

extracts with a combination of acetyl phosphate and formate. Keto acid was determined colorimetrically by the method of Friedemann and Haugen (22), which is fairly specific for pyruvate.

SUMMARY

1. The formation of a labile phosphate compound as the oxidation product of pyruvic acid with a bacterial enzyme system is described.

2. The compound is identified as monoacetyl phosphate by isolation of the silver salt in pure state.

3. The enzymatic transfer of phosphoryl groups between acetyl phosphate and adenylic acid is studied. The reversibility of the reaction is demonstrated through the observation of a phosphorylation of acetic acid by adenyl pyrophosphate as phosphate donor.

BIBLIOGRAPHY

1. Lipmann, F., *Nature*, **143,** 281 (1939).
2. Lipmann, F., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7,** 248 (1939).
3. Lipmann, F., and Tuttle, L C., *J. Biol. Chem.*, **153,** 571 (1944).
4. Lipmann, F , *J. Biol. Chem.*, **134,** 463 (1940).
5. Breed, R. S., Murray, E G. D., and Hitchens, A. P., Bergey's manual of determinative bacteriology, Baltimore, 5th edition, 307 (1939).
6. Davis, J. G., *Biochem. Z.*, **267,** 357 (1933)
7. Lipmann, F., *Enzymologia*, **4,** 65 (1937).
8. Koepsell, II. J., and Johnson, M. J., *J. Biol Chem* , **145,** 379 (1942).
9. Warburg, O., *Biochem. Z* , **152,** 51 (1924).
10. Clift, F. P., and Cook, R P , *Biochem J* , **26,** 1788 (1932)
11. Fiske, C. H , and Subbarow, Y , *J Biol. Chem* , **66,** 375 (1925).
12. Lohmann, K., and Jendrassik, L., *Biochem Z* , **178,** 419 (1926).
13. Needham, D M., *Biochem J.*, **36,** 113 (1942)
14. Baer, E., *J Biol. Chem* , **146,** 391 (1942).
15 Fieser, L F , Experiments in organic chemistry, New York, 332 and *cf.* Fig. 55 (1941).
16 Lipmann, F , in Nord, F F , and Werkman, C. H., Advances in enzymology and related subjects, New York, **1,** 99 (1941).
17. Meyerhof, O , Ohlmeyer, P , and Moehle, W., *Biochem Z.*, **297,** 113 (1938)
18 Kalckar, H M., *J. Biol. Chem* , **148,** 127 (1943)
19 Lewis, G. N , and Randall, M , Thermodynamics and the free energy of chemical substances, New York (1923).
20. Utter, M. F , and Werkman, C H., *Arch. Biochem.*, **2,** 491 (1943).
21. Kubowitz, F., *Biochem Z.*, **274,** 285 (1934).
22. Friedemann, T E , and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943).

A METHOD FOR THE DETERMINATION OF FIVE-TENTHS TO TWO MILLIMICROGRAMS OF RIBOFLAVIN

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A need rose in this laboratory for the determination of the riboflavin in the cornea of the rat. Since there are but 4 or 5 millimicrograms (10^{-9} gm.) of riboflavin present in each cornea, existing methods were found inadequate. The success of the microbiological method of Snell and Strong (1) for the determination of amounts of riboflavin of the order of 100 millimicrograms suggested the possibility of obtaining the required sensitivity by a micro modification of this procedure. After numerous modifications it has been possible to develop a reliable and practical method for measuring 0.5 to 2.0 millimicrograms of riboflavin with an over-all precision of 3 to 5 per cent, which is as good as or better than can be obtained with the macromethod.

Pennington *et al.* (2) suggested reducing the volume of growth medium from 10 ml., as used in the original procedure, to 0.2 ml. and allowing growth to occur in the individual depressions of spot plates. In our hands, failure was regularly encountered in the use of this micro modification. The substitution of individual serological tubes for the spot plate depressions led to some moderately successful assays, with frequent failures and erratic results. Furthermore, the amount of acid produced per unit of riboflavin was considerably less at the 0.2 ml. volume than at the standard 10 ml. volume, which decreased the sensitivity. These difficulties were therefore investigated and, as a result, it has become possible to modify the conditions of growth of *Lactobacillus casei* so that entirely consistent results may be regularly obtained with the order of 1 millimicrogram of riboflavin in a volume of 0.2 ml. That this is by no means the limit of sensitivity is shown by the fact that good results have been obtained with 0.1 to 0.5 millimicrogram of riboflavin in an incubation volume of 0.05 ml. 4 γ of liver, perhaps 400 cells, contain 0.1 millimicrogram of riboflavin.

Method

Reagents and Materials—The medium used for growing the *Lactobacillus casei* for inoculation and the riboflavin-free basal medium are the same as described by Snell and Strong, except that potassium acetate is added to

make the former 0.15 M in potassium acetate and the latter, 0.3 M.   Since the basal medium is finally diluted 1:1, the final concentration of potassium acetate in this medium is likewise 0.15 M.   In addition to the potassium acetate, 0.5 ml. of 0.4 per cent cysteine solution is added to each 10 ml. of basal medium just before inoculation.   A few ml. of cysteine solution are freshly made each time from either cysteine and water, or cysteine hydrochloride with the calculated amount of NaOH.   The solution is boiled just before use and added to the sterile basal medium with a sterile pipette.

The assays are carried out in 0.75 ml. serological tubes, 6 × 50 mm. These are cleaned routinely by boiling briefly in half concentrated nitric acid and then boiling in distilled water.   This avoids the use of chromic acid.   The washed suspension of *Lactobacillus casei* is prepared as usual; we have obtained most consistent results from an 18 to 24 hour culture made from a work stab not over 1 week old.

Procedure

To the tissue sample containing 2 to 8 millimicrograms of riboflavin is added 0.1 ml. of 0.1 N HCl, either with a 0.2 ml. graduated pipette, the end of which has been drawn out to a slender tip, or better, with a Levy-Lang constriction pipette (3).   The tubes are plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure, with care thereafter to protect from light, especially while still hot.   The weight of a few tubes before and after autoclaving should be determined to see that no significant change occurs in the volume.   After cooling, exactly 0.3 ml. of 0.030 N NaOH is added with a slender tipped 1 ml. graduated pipette, or a constriction pipette, and each tube is promptly and thoroughly mixed by twirling in the tube a slender rod with a hooked end.   This leaves the solution with an excess acid concentration of about 0.002 M.   Three 0.1 ml. aliquots are transferred to 0.75 ml. serological tubes (6 × 50).   At the same time, tubes for establishing a standard curve are prepared by measuring out 0.1 ml. in triplicate of each of four standards and a blank.   The standards contain 0.5, 1.0, 1.5, and 2.0 millimicrograms of riboflavin per 0.1 ml. or 0.5, 1.0, 1.5, and 2.0 γ per 100 ml., and are made up in 0.002 N HCl.   The blank is 0.002 N HCl.

All the tubes are plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure.   The whole rack should be wrapped in black cloth during autoclaving and until thoroughly cooled.

The tubes are inoculated as suggested by Pennington *et al.* (2), by pipetting into each tube 0.1 ml. of basal medium previously inoculated with 2 drops of a washed suspension of *Lactobacillus casei* per 10 ml.   The suspension of bacilli is prepared as in the regular Snell and Strong procedure. The medium is added with a 1 ml. sterilized pipette, the tip of which is

drawn out to a slender point and slightly bent. The volume of basal medium must be delivered with an accuracy of 2 to 3 per cent for best results. The cotton plug is replaced and each tube is mixed by tapping with the finger. Flaming of the mouth of the tube is unnecessary.

The rack of tubes is placed in a vacuum desiccator containing damp cotton swabs, and the air therein replaced with CO_2 by alternately reducing the pressure to about 150 mm. of Hg and introducing CO_2 back to atmospheric pressure. After this procedure is repeated four or five times, the pressure is left at about 700 mm. of Hg and the desiccator is placed in an incubator at 38° for 3 days.

The tubes are then removed from the desiccator. A minute droplet of caprylic alcohol is added to each tube to prevent foaming and the CO_2 is blown off by carefully aerating each tube with a capillary tube of not over 0.5 mm. diameter. The bubbles should be small enough so that spattering is entirely confined to the tube. After aeration for 1 minute, a 0.02 ml. drop of 0.04 per cent brom-thymol blue is added and the tube is titrated with 0.3 N NaOH from a 0.2 ml. Rehberg burette. Stirring is accomplished by bubbling with air. Neither the air bubbler nor the burette tip should be over 0.7 mm. in diameter. Since the indicator is soluble in caprylic alcohol, the amount of caprylic alcohol used should be kept small, and bubbling during titration should not be too prolonged. While one tube is being titrated, the next tube is being aerated, so that the preliminary aeration does not add to the time involved.

The results are calculated from the standard curve, just as with the microbiological assay on 10 ml. However, with some tissues the results are influenced by the presence of substances other than riboflavin in the tissue extract. The following procedure appears to obviate this difficulty in a most satisfactory manner, and should be used for highest precision if conditions will permit. Tissue of the type being analyzed is extracted, etc., exactly as described above, but on a larger scale. The final extract in 0.002 N HCl is irradiated for 30 minutes in a Pyrex tube at a distance of 3 or 4 cm. from a General Electric HB-4 mercury arc lamp to destroy the riboflavin present. With as little dilution as possible, standards are prepared from this extract containing 0, 0.5, 1.0, 1.5, and 2.0 millimicrograms of riboflavin per 0.1 ml. To produce the standard curve, these standards are used in place of pure riboflavin solutions.

DISCUSSION

The changes made in adapting the Snell and Strong method to the 0.2 ml. level were introduced as a result of observing that (a) riboflavin is partially destroyed during autoclaving in small tubes at pH 7.0, (b) air is inhibitory to *Lactobacillus casei*, (c) CO_2 stimulates the growth and acid pro-

duction of this organism, (d) buffers in the pH range from 4 to 6 stimulate growth and acid production, (e) on standing after autoclaving, the basal medium may become defective and it can be restored in effectiveness with cysteine.

Destruction on Autoclaving—Although riboflavin is ordinarily considered stable to autoclaving between pH 7.0 and 1.0, it was found that when small tubes were used there was a marked destruction of riboflavin on autoclaving in distilled water. This was probably due to the alkali surface layer of soft glass. This loss was avoided by the routine cleaning of the small soft glass tubes in boiling nitric acid and by adjusting the extracts and standard riboflavin solutions to an HCl concentration of 0.002 N.

Inhibition by Air and Stimulation by CO_2—The factor which was found to be most disturbing to assays on a 0.2 ml. scale was the presence of air. The *Lactobacillus casei* appears to be inhibited by even moderate tensions of oxygen. This effect is obscured in large tubes, where the large inoculum and subsequent large bulk of organisms undoubtedly keep the oxygen ten-

TABLE I

Influence of Gas Phase on Acid Production

The acid values represent c.mm. of 0.3 N acid produced with 1.1 millimicrograms of riboflavin.

CO_2, %	100	98	80	0	0	Air
N_2, %	0	0	0	100	80	
O_2, %	0	2	20	0	20	
Acid produced	29.1	24.9	21.0	21.3	17.4	19.0

sion low in the deeper layers of the tubes. Furthermore, the depth of liquid is 4 or 5 times greater in the large tubes than in the small ones, which would decrease the penetration of oxygen into the deeper layers. In Table I is shown the acid production in the presence of air, CO_2, and N_2 with 1.1 millimicrograms of riboflavin in a 0.2 ml. volume. Compared with the results in air, there is a marked stimulation by CO_2 and slight stimulation by nitrogen. In similar experiments, in which the tubes were incubated in air, shaking during growth produced additional inhibition, whereas there was no inhibition due to shaking when incubation took place in CO_2.

Stimulation by Added Buffer—In confirmation of the findings of Stokes and Martin (4) it was observed that the addition of extra acetate to the basal medium increases both growth and acid production with a given amount of riboflavin. This appears to be due to the increased buffer capacity of the basal medium, since a variety of other buffers in the region of pH 4 to 6, *e.g.* propionate, butyrate, acid phthalate, and pyridine, likewise increased the production of acid. Acetate proved most satisfactory, and

potassium acetate was chosen since larger amounts of the potassium salt than of the sodium salt were tolerated by the bacteria before inhibitory effects appeared.

Old Media—In spite of the substitution of CO_2 for air in the microbiological assay, with resultant marked improvement in results, occasional assays failed. This failure was traced to the use of basal media which had been autoclaved and then allowed to stand for some time in the ice box before use. This trouble was completely overcome by the addition of cysteine to the medium just before use. Additional cystine or thioglycolic acid helped somewhat but neither was as effective as cysteine. Reboiling the medium just before use did not result in improvement.
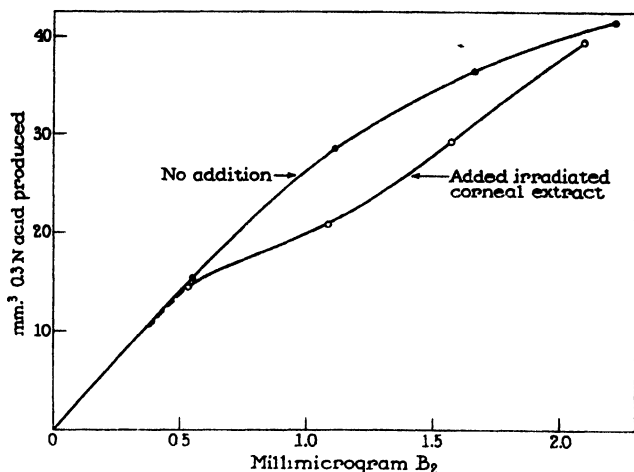
FIG 1 Standard curve of acid production *versus* riboflavin

Additional Factors—The evaporation of water during incubation is relatively much greater from the small tubes containing 0.2 ml. of solution than from large tubes containing 10 ml. If uncontrolled, this evaporation will produce low and erratic results. This effect is easily prevented with tubes grown in an enclosed chamber by the introduction of wet cotton swabs into the chamber.

It is likely that almost all tissue extracts, no matter how prepared, influence in one degree or another the amount of acid produced for a given amount of riboflavin. It has been repeatedly shown that, unless lipids are removed, a marked stimulation of acid production will result (5, 6). The removal of lipids, however, does not completely solve the problem. In Fig. 1 is shown the result of adding riboflavin-free (irradiated) corneal extract to a series of tubes, forming a standard curve of acid production *versus*

riboflavin. The riboflavin-free extract was prepared as described above and probably contained little or no lipid. Extract equivalent to 0.2 mg. of cornea was added to each tube.

It will be seen that the influence of the tissue extract on acid production depends on the amount of riboflavin present. The influence varies from 0 with 0.5 millimicrogram of riboflavin per tube to an inhibition of 18 per cent with 1.3 millimicrograms of riboflavin. To show whether or not the ultraviolet light used in the destruction of riboflavin produced an aberration in this curve, known amounts of riboflavin were added to non-irradiated corneal extract. The recovery calculated from the lower curve in Fig. 1 was within the limits of error of the method.

As a result of the foregoing observations the procedure detailed above was adopted, and it has given uniformly consistent results. In Table II is

TABLE II

Reproducibility of Titration with Pure Riboflavin Solutions

Riboflavin	Average* titration	Standard deviation	Coefficient of variation	P E M †
millimicrograms	*c mm*	*c mm*		
0.55	15.6	0.4	2.9	1.2
1.10	27.8	0.3	1 7	0.7
1.65	36.1	0.5	2 3	0.9
2.20	42.4	0.7	3.0	1.2

* C.mm. of 0 3 N NaOH corrected for the blank.

† Probable error of the mean, expressed as per cent of the mean, for an assay in triplicate.

shown the reproducibility for pure riboflavin solutions as evidenced by six consecutive standard curves. Each point in each curve was made in triplicate, giving a total of eighteen determinations at each level. The standard deviation of a single determination has been calculated at each riboflavin level from the deviations within each individual set of three. The coefficient of variation on these pure solutions is actually smaller than for a similar series of standard curves with 10 ml. volumes and following the regular Snell and Strong procedure.

In the analysis of biological materials the variability is greater than with pure solutions. From a series of twenty-four corneal extracts in triplicate, with 1.0 to 1.5 millimicrograms per tube, the coefficient of variation was found to be 8 per cent for a single determination, which is equivalent to a probable error of the mean of 3 per cent for an assay in triplicate. This again compares favorably with the macromethod.

SUMMARY

A modification of the microbiological method of Snell and Strong has been described for the determination of 0.5 to 2.0 millimicrograms of riboflavin. The probable error for assays made in triplicate is about 1 per cent for pure riboflavin solutions and about 3 per cent for the particular biological material tested (rat cornea).

The reasons for making certain changes in adapting the original procedure to the determination of these smaller amounts of riboflavin are discussed.

BIBLIOGRAPHY

1. Snell, E. E., and Strong, F M , *Ind. and Eng Chem., Anal. Ed* , **11,** 346 (1939)
2 Pennington, D. E., Snell, E. E , Mitchell, H K , McMahan, J R , and Williams, R. J., *Univ Texas Pub* , *No 4137,* 14 (1941).
3. Levy, M., *Compt -rend. trav. Lab. Carlsberg, Série chim* , **21,** 101 (1936).
4. Stokes, J. L., and Martin, B B., *J. Biol Chem.,* **147,** 483 (1943).
5. Bauernfeind, J C., Sotier, A L , and Boruff, C. S., *Ind and Eng. Chem., Anal. Ed.,* **14,** 666 (1942).
6 Strong, F. M., and Carpenter, L E , *Ind and Eng Chem., Anal Ed.,* **14,** 909 (1942).

USE OF THE MACRO FERMENTATION METHOD FOR THIAMINE ASSAY*

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The yeast fermentation method of Schultz, Atkin, and Frey (1–3) has been widely used for the assay of thiamine. When the method was tried in this laboratory for the assay of meat and eggs, three sources of error were observed.

First, the range of linear response of the yeast to graded amounts of thiamine may vary with the substance assayed and the yeast used.

Second, the activity of crystalline thiamine is greater in the presence of the other constituents of the food samples than it is in the basal medium alone. Since the standard in the original method is taken to be the volume of gas produced by the yeast under the stimulus of a known amount of thiamine added to the basal medium, there is a tendency for the assay values to be too high.

Finally, as pointed out by Deutsch (4), the sulfite cleavage procedure does not completely destroy the activity of thiamine in stimulating the carbon dioxide production of yeast. The effect of these three sources of error upon actual assay values has been studied by repeated assays of food samples. A standard procedure has been developed which eliminates these potential errors in the fermentation method.

EXPERIMENTAL

The fermentometer used in this laboratory is patterned after the original fermentometer described by Schultz and Landis (5). The basal medium consisted of Solution A (buffer and niacin) and Solution B (dextrose and salts) as suggested by Schultz *et al.* (3). Fleischmann's commercial bakers' yeast was obtained weekly in half pound cakes and stored in a stoppered flask at 5°. Thiamine standard solution and yeast suspension were prepared as directed (3). In each of the six 250 cc. reaction bottles were placed 5 cc. of Solution A, 15 cc. of Solution B, and the substance assayed. The total volume was made up to 75 cc. with distilled water and 25 cc. of the yeast suspension added. All pH adjustments were made with a Beckman model F pH meter.

Samples which could be obtained as a fine powder (bread, lyophilized

meat) were weighed dry and diluted with distilled water as desired. Moist solid samples (fresh meat) were ground as finely as possible with a meat grinder, weighed, diluted with distilled water, and mixed in a Waring blendor. Fluid samples (egg) were blended and a known weight of this mixture diluted to volume. The sulfite cleavage for the destruction of thiamine in aliquots of the sample assayed was carried out as suggested by Schultz et al. (2, 3). The aliquot thus prepared is referred to as the blank. Samples were adjusted to pH 5.2, heated in a boiling water bath for 30 minutes, and readjusted to pH 6.2, as directed by Schultz et al. (2, 3) so that they might receive the same treatment as the blank.

The fermentation method for thiamine assay depends upon the fact that the response of yeast to added thiamine as measured by carbon dioxide production is linear over a definite range. Schultz et al. (1, 2) in their description of the method found the response to be linear between 2 and 4 γ and used these amounts of crystalline thiamine as their standards.[1] The thiamine was added directly to the basal medium alone. The difference in carbon dioxide production measured volumetrically in 3 hours was assumed to represent the stimulating effect of 2 γ of thiamine (4 − 2 γ). At the same time a sample and a blank were prepared in a similar manner and the difference in gas production between them taken to indicate the increase in carbon dioxide production due to the thiamine in the sample. The actual thiamine content of the sample was then calculated by a simple proportion.

Although this procedure has yielded excellent results for cereal products, we did not find it satisfactory for egg assays. Further study revealed that the relationship between the thiamine in the presence of the other egg constituents and the volume of carbon dioxide produced in 3 hours was not linear beyond a concentration of 2 γ of the vitamin. In fact, the response of the yeast used in the egg assay series of 1943 was not linear beyond 1 γ of thiamine. These facts are presented graphically in Fig. 1.

This experience with egg samples demonstrated that the range over which the response to thiamine remains linear varies with the substance being assayed and with the yeast used. This range was not found to be as restricted with other substances studied. However, in no experiment did the response remain perfectly linear when more than 3.5 γ of thiamine were added (Fig. 2). Thus routine use of the 2 to 4 γ range suggested by Schultz et al. (1-3) would have produced significant errors in our assay values for all substances and resulted in serious errors in the case of egg samples. For this reason, we believe that it is necessary to make

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[1] This is equivalent to a 1 to 2 γ range on their new fermentometer in which half the previous quantities of all solutions (3) is used. It cannot be directly compared to the assay range used in the micro fermentation methods.

a preliminary run, adding graded amounts of crystalline thiamine to the blank of each new type of material studied, in order to determine the assay range.

When we began to assay various types of food samples, a second factor was found which could produce variation in the values obtained. Schultz *et al.* (3) suggest the addition of standard thiamine to bring low blanks within the same range of carbon dioxide production as the samples. After we observed the relation between thiamine and carbon dioxide production to be linear to zero, it became apparent that the addition of thiamine
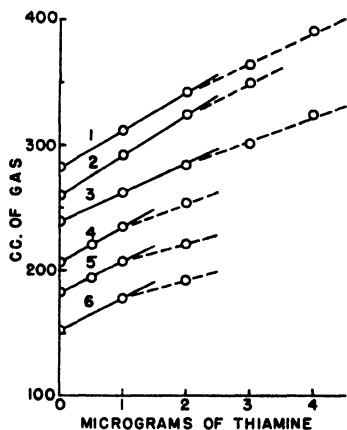
FIG. 1   FIG. 2

FIG. 1. Effect of egg blank on response of yeast to added thiamine. In addition to the basal medium 1 gm. of sulfite-treated whole egg has been added to each reaction bottle. Curves 1 to 3 represent runs made in the spring, 1944. Curves 4 to 6 were determined in the summer, 1943.
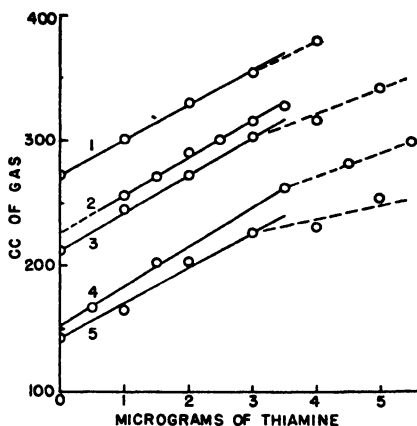
FIG 2 Response of yeast to graded amounts of crystalline thiamine In addition to the basal medium 1 gm. of lean beef (the results shown in Curve 1) and 0.05 gm. of sulfite-treated liver concentrate (Curve 5) were added to the respective reaction bottle.

to sulfite blanks for this purpose should be, in theory at least, an optional procedure.

In a series of trials in which we ran both types of blanks and calculated the values for thiamine content of the samples involved, consistent and generally striking differences were obtained. The values obtained when thiamine was added to the blank were always lower than those found when this addition was not made (Table I). However, when the activity of thiamine added to the blank was itself used as a standard (new standard), the resulting values agreed well with those obtained by the use of the old standard and thiamine added to the blank to bring it within the range of

the other bottles. In the case of bread, this discrepancy is small but for a highly concentrated yeast extract is seen to be very large. The values listed in Table I which are based on the old standard were found to be about 10 per cent too high for Staff bread, 20 per cent and above for whole egg, 30 per cent for lean beef, and nearly 100 per cent for yeast extract. Use of the old standard is thus seen to introduce great errors into the assay of some foods although relatively small ones in the study of others.

The true cause of this discrepancy becomes obvious from a consideration of Table II. It is due to the fact that thiamine is more effective in stimu-

TABLE I

Effect of Choice of Standard on Assay Value and Results of Adding Thiamine to Bring Blank within Assay Range

Substance assayed	Weight of sample	Assay value, old standard*	Assay value, new standard†	Assay value, old standard, blank with added thiamine‡
	gm.	*γ per gm*	*γ per gm*	*γ per gm*
Cooked lean beef, Sample 16	2.00	1 29	1 01	1.02
" " " " 22	1.00	0.86	0.62	0.62
Staff bread, Sample 1	0.50	2.86	2.60	2.68
Yeast extract	0.005	1004	560	374
" "	0.0025	1184	580	352
Egg yolk, Sample 5	1.0	44.5	28.8	33.6
" " " 5	0.6	36.4	29.8	29.2
" white, " 4	4.25	1.0	0.0	0.0
Whole egg, " 3	0.8	13.5	9.6	
" " " 2	2.0	10.8	8.6	8.3
" " " 2	2.0	10.3	8.8	8.6

* The old standard represents the volume of gas produced by 2 γ of thiamine $(4 - 2 \gamma)$ added to the basal medium alone.

† The new standard is the volume of gas produced by 2 γ of thiamine similarly added to the blank.

‡ The old standard has been used but 2 γ of thiamine have been added to the blank to bring it into the range of the other values.

lating carbon dioxide production of yeast in the presence of either the sample or the blank than it is when added to the basal medium alone. This means that, unless this effect is small, crystalline thiamine added to the basal medium alone cannot serve as a reliable standard. The fact that this effect is small for bread (12 per cent), but greater for meat and egg (21 and 23 per cent), is again shown in Table II. Furthermore, this effect varies from sample to sample of the same type of food (*cf.* 35 and 18 per cent for egg yolk samples at different concentrations). In the case of yeast samples it seems to be greatly influenced by the concentration of the sample, as values of −6, 38, and 58 cc. for the response to 2 γ for thiamine at various dilutions of the sample (0.001 to 0.10 gm.) illustrate.

This effect was not further investigated. More important, however, is the fact that the addition to the blanks of the same amount of thiamine as is taken for the standard automatically compensates for the above effect.

In our early meat assays we used a 2 γ (2 to 4 γ) range as a standard and also added 2 γ of thiamine to the blanks. This thiamine was added to the basal medium alone, and the resulting volume of gas, taken as the standard for calculation of thiamine content, was too small. It was too small because the same amount of thiamine in the sample would permit much larger gas production. However, this small value was subtracted

TABLE II

Volume of Gas Produced under Stimulus of 2 γ of Thiamine for Old and New Standards

Blank prepared from	Weight of blank	Gas, old standard*	Gas, new standard*	Differ-ence†
	gm.	*cc.*	*cc*	*per cent*
Cooked lean beef, Sample 16	2 0	104	82	21
" " " " 22	1.0	64	49	23
Staff bread, Sample 1	0.5	60	53	12
Yeast extract	0.005	56	31	45
" "	0 0025	51	25	51
" "	0.01	58	37	46
" "	0 025	43	37	14
" "	0 10	−6 1 run (conditions		
" "	0.01	38 and yeast iden-		
" "	0 001	58 tical)		
Whole egg, Sample 3	2 0	64	51	20
" " " 4	2.0	68	58	15
" " " 1	0.8	74	55	26
" " " 1	2.0	72	55	24
Egg yolk	1.0	68	44	35
" "	0.6	67	55	18
" white	4.25	61	36	41

* The use of the terms old and new standard is the same as in Table I.

† As in Table I the discrepancy between the old and new standard is increased slightly by use of a 2 to 4 γ range (*cf.* Fig. 2).

from the gas produced by the blank plus 2 γ to give the supposed true blank. This volume assumed to represent the true gas production of the ingredient of the blank was too high. The two false values will be found to cancel each other exactly. The last column in Table I shows the effectiveness of this compensation. For example, lean beef Sample 16 appears to have 1.29 γ of thiamine per gm. by the old standard but only 1.01 γ per gm. when calculated according to the new standard. Adding thiamine to the blank corrects the former values to 1.02 γ per gm., almost identical to the value obtained by the use of the new and presumably correct standard.

The discrepancy between the old and new standards was also eliminated by the addition of 0.05 gm. of sulfite-cleaved liver concentrate (Wilson 1:20 liver concentrate paste) to each of the reaction bottles. The concentrate seems to supply an excess of whatever stimulating factors the meat and egg blanks possess to all of the bottles. This effect of liver in reducing the differences in thiamine activity due to greater stimulation in gas production in the presence of the blank or sample is shown (Table III). For example, in a typical run with liver present, 47 cc. of gas are produced by the old standard and 49 cc. by the new as compared to 47 and 37 cc. when no liver is added.

Since Deutsch (4) using the micromethod has clearly shown that the inactivation of thiamine by sulfite cleavage is not necessarily complete, it seemed desirable to investigate the effectiveness of cleavage under the

TABLE III

Agreement between Old and New Standards When Liver Extract Is Added to Basal Medium

Sample No. (2 0 gm whole egg)	Liver* extract per bottle	Effect on standard			Effect on assay value		
		Old standard†	New standard†	Difference	Assay value by old standard†	Assay value by new standard†	Assay value, old standard, and thiamine added to blank
	gm	*cc*	*cc*	*per cent*	*γ per gm*	*γ per gm*	*γ per gm*
6	0.05	47	49	4	5.5	5 3	5.1
7	0 025	69	67	3	7.4	7.5	7.5

* Sulfite-cleaved Wilson's 1:20 liver concentrate paste was added to all reaction bottles.

† The use of the terms old and new standard is the same as in Table I.

conditions of our assay. For the substances and concentrations studied, the sulfite cleavage process was found to be very satisfactory. On the average, less than 5 per cent of the activity originally found for the sample remained. Activity greater than 8 per cent was not encountered in the small series of sulfite-cleaved samples studied. The effectiveness of the cleavage procedure can be readily determined for each type of substance studied and the strain of yeast used, and assay runs can be corrected for this factor if desired.

DISCUSSION

We have found that the assay range for each type of substance assayed should be determined in a preliminary fermentometer run. Furthermore, the assay procedure itself must at times be modified to take into consideration the greater activity of thiamine in the presence of the sample or blank itself. Is this enhanced activity due to the failure of the basal medium alone to supply in excess all the other factors which stimulate

carbon dioxide production? Certainly something supplied by the sample and its blanks can potentiate the action of thiamine. The effect of this factor or factors varies greatly with the nature of the sample.

One approach to this problem would be to study more completely the components of the medium specifically required in the assay procedure. The experiments with liver concentrate cited above suggest that a more nearly complete basal medium would effectively eliminate this potential error. The experiments with liver concentrate were not continued, however, because we believed it safer and simpler to be sure that the same factors which operate in the sample will be at work in the standard.

This can be done by adding the thiamine directly to the blank, since the data reveal that whatever the factor or factors are which potentiate the action of thiamine, they are not destroyed by sulfite cleavage. Accordingly we have adopted the procedure of determining the range of the linear response to thiamine in the presence of the material to be assayed and confirming its extension to zero. To secure a standard for comparison with the activity of the sample, we add a suitable amount of thiamine directly to the blank.

This means that one complete assay run contains three bottles: blank, blank plus a known amount of standard thiamine, and sample. The difference in gas production between the blank and the blank plus thiamine gives the carbon dioxide-stimulating effect of the known quantity of thiamine. Similarly the difference between the blank and sample gives the added gas production due to the thiamine in the sample. The actual amount of thiamine is calculated by simple proportion. Figs. 1 and 2 show standardization curves for this procedure.

The above application of the method would seem to give the best measure of the true thiamine activity of the sample. It gives consistent results even when the difference between the two types of standards is varying from sample to sample. This is shown best in the egg assays (Table I) in which values of 28.8 and 29.8 γ per gm. are obtained for egg yolk by this method, when the original method gave 44.5 and 36.4 in simultaneous runs. This method also provides that if inhibitors are present (as is suggested by some of the yeast standards in Table II) they will act equally upon both the thiamine of the sample and that of the standard.

Deutsch (4) has discussed the biochemistry of the yeast fermentation method and studied the effectiveness of the sulfite cleavage process. Although his data and those of Schultz et al. (3) show that the sulfite cleavage products of thiamine may have considerable fermentation-stimulating activity under some conditions, we have not found these to be troublesome under the conditions of the macro fermentation assay for the substances studied here. The per cent inactivation by sulfite depends in part on the batch of yeast (4), the substance treated and its concentrates

(3), and probably the total sugar content of the reaction mixture (6).   We believe, therefore, that the effectiveness of the cleavage process should be determined for each type of food substance under the local assay conditions.   A correction can then be made when it would be of significance.

Among other factors which were investigated as sources of error for the method and found to be of no significance are variations in the absolute amount of gas produced by similar samples and standards in different runs (due to age and condition of the yeast), variations in pressure due to infrequent leveling of the fluid columns in the eudiometers, brief interruptions in shaking, and variations of as much as 3°.

SUMMARY

When the fermentation method for the assay of thiamine was used for the assay of meat and egg products, three sources of error were found to be significant.   The range of linear response to added thiamine varies with the sample assayed and the yeast used.   Thiamine added to the basal medium alone shows less activity than it would in the presence of the constituents of the sample or blank.   The sulfite cleavage procedure does not necessarily destroy all of the activity in the blank.   These potential errors can be eliminated by the following modifications or precautions.

1. A preliminary run is made for each type of substance assayed in which graded amounts of crystalline thiamine are added to the blank.   This determines the range over which the response to thiamine is linear.

2. A standard three bottle assay procedure (run in duplicate if desired) in which one bottle contains the sample, one the blank alone, and the third the blanks plus a suitable amount of crystalline thiamine as determined above is carried out.

3. The amount of gas produced by the known amount of thiamine in the presence of the blank is used for the calculation of the thiamine content of the sample

4. The effectiveness of the sulfite cleavage of a known amount of thiamine added to the blank is determined for each type of substance assayed.   A correction for the activity of the cleavage products is made if necessary.

BIBLIOGRAPHY

1. Schultz, A. S., Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, **59,** 948, 2457 (1937); **60,** 1514, 3084 (1938).
2. Schultz, A. S , Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, **63,** 632 (1941).
3. Schultz, A. S., Atkin, L , and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed* , **14,** 35 (1942).
4. Deutsch, H. F., *J. Biol. Chem.*, **152,** 431 (1944).
5. Schultz, A. S., and Landis, Q., *J. Am. Chem. Soc.*, **54,** 211 (1932).
6. Lanen, J. M., Broquist, H. P., Johnson, M. J., Baldwin, I. L., and Peterson, W. H., *Ind. and Eng. Chem., Ind. Ed.*, **34,** 1244 (1942).

α-KETOGLUTARIC DEHYDROGENASE OF ANIMAL TISSUES*

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α-Ketoglutaric acid is an important intermediate in the metabolism of carbohydrate, protein, and fat in cells. It is formed from carbohydrate via pyruvic and isocitric acids, from glutamic acid by oxidative deamination or transamination, and from fatty acids via the enzymatic condensation of oxalacetate with β-keto acids to citric acid (1). α-Ketoglutaric acid is readily oxidized to $CO_2 + H_2O$ by most animal cells but it undergoes oxidative decarboxylation to succinic acid $+ CO_2$ (Reaction 1) when the tissue succinic dehydrogenase is blocked by malonate (*cf.* (2)).

$$(1) \quad COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH + 0\,5O_2 = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CO_2$$
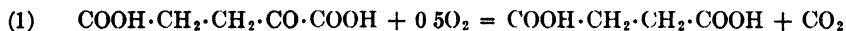
The present work was undertaken in order to obtain information on the mechanism of oxidation of α-keto acids in animal tissues and as a preliminary step in the investigation of the mechanism of pyruvate oxidation. In animal cells this reaction involves condensation with dicarboxylic acids and is undoubtedly more complicated than the oxidative decarboxylation of pyruvate catalyzed by some bacterial enzymes (3–5) or that undergone by α-ketoglutarate in tissue preparations.

The enzyme catalyzing Reaction 1, which will be referred to as α-ketoglutaric dehydrogenase (6), is present together with a number of other respiratory enzymes in the particles which one obtains in suspensions prepared from tissues by thorough grinding with sand and saline or phosphate solutions and centrifuging the mixtures at low speeds. There is evidence that the α-ketoglutaric dehydrogenase is a diphosphothiamine enzyme (7). It will be shown in this paper that the activity of the enzyme is coupled with phosphorylation of phosphate acceptors, that it is dependent on the presence of inorganic phosphate, magnesium ions, and adenine nucleotide,[1] and that cytochrome *c* is a physiological hydrogen (electron) carrier between α-ketoglutarate and molecular oxygen.

Oxidative Decarboxylation of α-Ketoglutarate and Its Coupling with Phosphorylation—Preparations of a very active α-ketoglutaric dehydrogen-

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[1] The term adenine nucleotide is used in this paper to indicate both adenosine-5-monophosphate (muscle adenylic acid) and adenosine-5-triphosphate.

ase can be obtained from cat heart and have been used throughout this work. In the presence of high concentrations of malonate (0.025 to 0.05 M) these preparations catalyze the aerobic oxidation of α-ketoglutaric acid according to Reaction 1; as much as 5 to 6 mg. of the acid can be utilized in 40 minutes at 36.5° with 1.5 cc. of the enzyme suspension. When heart enzyme is used with an excess of α-ketoglutarate, the rate of oxygen uptake remains constant for at least 20 minutes; after this time there is a gradual drop which is probably caused by enzyme inactivation. With pigeon brain preparations the rate of oxygen uptake shows a gradual drop from the

<div align="center">TABLE I</div>

Oxidative Decarboxylation of α-Ketoglutaric Acid and Glucose Phosphorylation

The manometer bottles contained 1.5 cc. of enzyme, 0.004 M MgCl₂, 0.025 to 0.03 M potassium phosphate buffer of pH 7 5, 0 0004 M adenosine triphosphate, and 0.05 M sodium malonate. Samples with pyruvate as substrate had 0.002 M fumarate but no malonate. The final volume was made up to 2 cc. with water. Temperature 36.5°. Gas phase 100 per cent oxygen. All values are corrected for enzyme blanks and are expressed in micromoles.

Experiment No.	Source of enzyme	Additions				Incubation time	Oxygen uptake	CO₂ evolution	R.Q	α-Ketoglutarate utilized	Succinate formed	Phosphate esterified	P:O ratio
		α-Ketoglutarate	Pyruvate	Glucose	NaF								
						min							
1	Cat heart	34				45	21.0	33.7	1.60	31.7	25.7		
2	" "	32				25	14.7	25.6	1.74	26.3	22.3		
3	" "	35		55	40	35	9.0	17.0	1.89	18.2	15.8	32.2	1.7
			54	55	40	19	14.0					44.5	1.6
4	" "	32		55	40	45	10.0	17.0	1.70	17.1	16.1	32.6	1.6
			54	55	40	25	16.0					45.8	1.4
5*	Pigeon brain†	30				35	8.2	14.3	1.74	15.7	11.5		

* 0.003 M adenosine triphosphate; 0.025 M sodium malonate.
† Homogenate. Dialyzed against 0.4 per cent KCl for 2.5 hours

beginning. The oxygen consumption in the absence of α-ketoglutarate is negligible after the minced tissue has been thoroughly washed with saline before extraction and it is nil if the enzyme suspensions from washed tissue have been dialyzed against saline or phosphate buffer.

In the presence of glucose the sugar is phosphorylated to hexose diphosphate with an uptake of 2 molecules of inorganic phosphate for every molecule of α-ketoglutarate oxidized to succinic acid + CO₂ (Table I). If exception is made of Experiment 1, in which owing to the large amount of α-ketoglutarate utilized malonate inhibition of the succinic dehydrogenase may have been rather incomplete, the experiments with cat heart enzyme

(Table I) give the following average molar ratios for every molecule of CO_2 produced: CO_2 evolved 1.00-oxygen uptake 0.56-α-ketoglutarate utilized 1.03-succinate formed 0.92-phosphate esterified 1.90; average R.Q. 1.8.

Since, as previously shown (8), dephosphorylation reactions which are not fully inhibited by fluoride interfere with phosphorylation and since, as shown in Table I, the P:O ratio, *i.e.* the ratio of atoms of phosphorus esterified to atoms of oxygen consumed, is the same for α-ketoglutarate and pyruvate (*cf.* (6, 8)), there is good reason for believing that the over-all reaction for the oxidative decarboxylation of α-ketoglutarate in the presence of glucose as catalyzed by the enzyme preparations used in this work can be expressed by Reaction 2.

(2) $COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH + 3H_3PO_4 + 1 5\ glucose + 0.5O_2 =$
$$COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CO_2 + 1.5\ hexose\ diphosphate$$
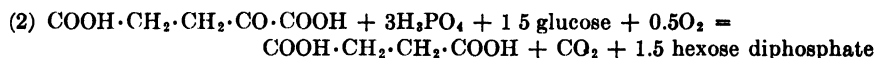
Oxidative Decarboxylation Versus Decarboxylation Plus Oxidation—The work of Green *et al.* (9), who obtained from animal tissues enzyme preparations that in the presence of diphosphothiamine and magnesium catalyzed the decarboxylation of α-keto acids, reopened the question as to whether the oxidative decarboxylation of α-keto acids is caused by one enzyme or by the combined cat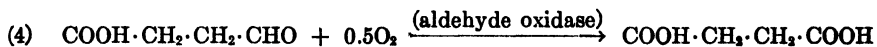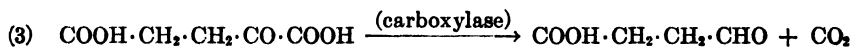alytic actions of a carboxylase and an aldehyde oxidase. In the case of α-ketoglutaric acid which was anaerobically decarboxylated to succinic semialdehyde (9), its oxidative decarboxylation (Reaction 1) might be the combined result of Reactions 3 and 4. It would seem, how-

(3) $COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH \xrightarrow{\text{(carboxylase)}} COOH \cdot CH_2 \cdot CH_2 \cdot CHO + CO_2$

(4) $COOH \cdot CH_2 \cdot CH_2 \cdot CHO + 0.5O_2 \xrightarrow{\text{(aldehyde oxidase)}} COOH \cdot CH_2 \cdot CH_2 \cdot COOH$

ever, that the α-ketoglutaric dehydrogenase is a distinct enzyme, since the preparations used in this work failed to catalyze either the anaerobic decarboxylation of α-ketoglutaric acid or the aerobic oxidation of (synthetically prepared) succinic semialdehyde at a rate comparable to that at which they catalyzed the oxidative decarboxylation of the former (Table II). Succinic semialdehyde did not affect the rate of oxidative utilization of α-ketoglutarate (Table II, Experiment 5). At present it is impossible to decide whether the one-step oxidative decarboxylation is brought about by the action of an enzyme complex containing two specific proteins or whether it is catalyzed by a single protein carrying two prosthetic groups of which one would be concerned with decarboxylation and the other with dehydrogenation.

Experiments 1 and 2 (Table II) also indicate that an anaerobic dismutation of α-ketoglutarate (10) is hardly if at all catalyzed by our enzyme preparations.

It might be pointed out that typical succinoxidase preparations did not act upon succinic semialdehyde, and competition experiments showed a lack of affinity between the succinic dehydrogenase and succinic semialdehyde, excluding the possibility of an oxidation of the latter to fumaric semialdehyde by succinoxidase. The small oxidation of the aldehyde which is found to occur in the heart preparations probably leads to the formation of some succinic acid, since succinic semialdehyde produced a catalytic stimu-

TABLE II

Comparative Utilization Rates of α-Ketoglutaric Acid and Succinic Semialdehyde under Anaerobic and Aerobic Conditions

The manometer bottles contained 1 5 cc. of enzyme and 0 004 M $MgCl_2$, plus 0.03 M potassium phosphate buffer of pH 7.5, 0 0004 M adenosine triphosphate, and 0.05 M sodium malonate (Experiments 1, 2, and 5), or 0.05 M phosphate buffer, 0.002 M adenosine triphosphate, and 0 025 M sodium malonate (Experiments 3 and 4). The final volume was made up to 2 cc with water   Temperature 36 5°. Incubation time 40 minutes   All values are corrected for enzyme blanks and are expressed in micromoles.

Experiment No.	Substrate	Gas	Oxygen uptake	CO_2 evolution	α-Ketoglutaric acid utilized	Succinic semialdehyde utilized
1	α-Ketoglutaric acid (56)	Nitrogen		2.2	7.2	
	" " (56)	Oxygen	23.8		39.3	
2	" " (38)	Nitrogen		2.0	3.4	
	" " (38)	Oxygen	22.2		37 1	
3*	" " (64)	"	9.0		20 2	
	Succinic semialdehyde (96)	"	0.2			1.0
4†	α-Ketoglutaric acid (64)	"	23.8		41 0	
	Succinic semialdehyde (96)	"	1.0			5.0
5	α-Ketoglutaric acid (39)	"	20.7		39 0	
	Succinic semialdehyde (100)	"	2 1			
	α-Ketoglutaric acid (39) + succinic semialdehyde (100)	"	23.2		39.0	

* Enzyme dialyzed 4 5 hours.
† Enzyme dialyzed 2 hours.

lation of the enzymatic oxidation of pyruvate similar to that caused by small amounts of succinate or fumarate.

Some Components of α-Ketoglutaric Dehydrogenase—As shown in Fig. 1, the enzyme depends for activity upon the presence of inorganic phosphate, adenine nucleotide, and magnesium ions, three of the substances which as previously shown (11) are also required for pyruvate oxidation.   Arsenate cannot be substituted for phosphate, a finding which is of interest in view of the fact that arsenate and phosphate are equally effective with bacterial (4, 5) and brain (12) pyruvic dehydrogenase.   Adenine nucleotide and

inorganic phosphate could be satisfactorily removed by dialysis against dilute potassium chloride solutions for 5 hours, whereas magnesium was removed to an adequate extent only by dialysis against pyrophosphate buffer of pH 8.5 for 2.5 hours followed by dialysis against salt solution for a similar period in order to get rid of the pyrophosphate.[2]   The effect of increasing concentrations of inorganic phosphate is shown in Fig. 2; maximum
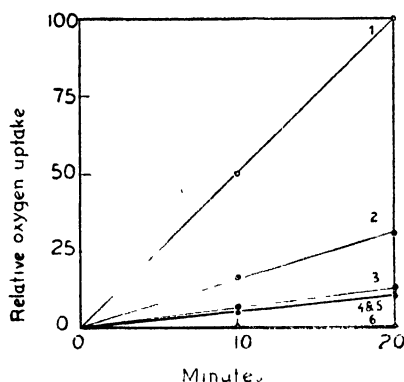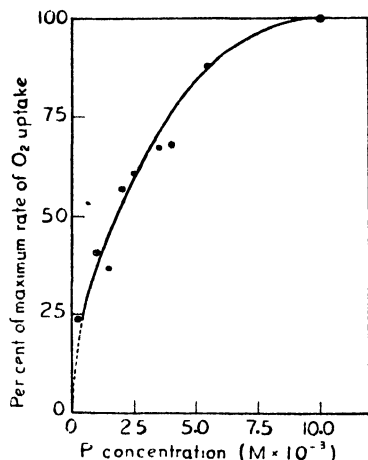
FIG 1   FIG. 2

FIG 1. Components of the α-ketoglutaric dehydrogenase (average results of two to seven individual experiments). The oxygen uptake of the samples in which one addition was omitted is expressed as a percentage of that of the complete system at a given time   The complete system (Curve 1) contained 1 5 cc. of dialyzed enzyme, MgCl₂ (0 004 M), phosphate buffer of pH 7.5 (> 0.01 M), adenylic acid or adenosine triphosphate (0.003 M), malonate (0.025 to 0.05 M), and α-ketoglutarate (0 015 to 0 03 M).   Curve 2, no magnesium (enzyme dialyzed successively against pyrophosphate and orthophosphate); Curve 3, arsenate instead of phosphate; Curves 4 and 5, either no phosphate or no adenylic acid; Curve 6, no α-ketoglutarate   Final volume 2 cc.; oxygen, 36 5°

FIG. 2 Rate of oxygen uptake as a function of the concentration of inorganic phosphate. The broken line represents extrapolation to zero phosphate concentration   The manometer bottles contained 1.5 cc. of dialyzed enzyme, 0.004 M MgCl₂, 0 005 M glycine buffer of pH 7.5, 0.003 M adenylic acid, 0.025 M malonate, and 0 03 M α-ketoglutarate.   Final volume 2 cc.; oxygen; 36.5°.

reaction rates were obtained with 0.01 M phosphate.   Extrapolation to zero phosphate concentration suggests that the system would be totally inactive in the complete absence of phosphate.   When both glucose and fluoride are added to a complete system containing an excess of α-ketoglu-

[2] The concentration of pyrophosphate left in the enzyme suspension was about 0.001 M.

tarate, there is often a sudden and very pronounced drop in the rate of oxygen uptake after 15 to 20 minutes, coinciding with the almost complete disappearance of inorganic phosphate from the reaction mixture.

Table III shows the effect of increasing concentrations of adenine nucleotide. While adenosine triphosphate seems to be more effective than muscle adenylic acid, it is apparent that rather high concentrations of nucleotide are required to produce maximum activity. However, it is possible that the nucleotides are partially destroyed by phosphatase and deaminase which may be present in the enzyme suspensions and adenylic acid would

<div align="center">

TABLE III

Effect of Adenine Nucleotide

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The manometer bottles contained 1.5 cc. of dialyzed enzyme, 0.004 M MgCl₂, 0.03 M potassium phosphate buffer of pH 7.5, 0.025 M sodium malonate, and 0.03 M sodium α-ketoglutarate. The final volume was made up to 2 cc. with water. Temperature 36.5°. Gas space 100 per cent oxygen. Incubation time 40 minutes.

Experiment No.	Additions		Oxygen uptake	α-Ketoglutaric acid utilized
	Compound	Amount		
		micromoles	*micromoles*	*micromoles*
1			1.4	4.4
	Muscle adenylic acid	1.2	3.0	6.1
	" " "	3.0	5.0	8.5
	" " "	6.0	17.4	32.0
2			1.3	10.0
	Adenosine triphosphate	0.4	4.3	12.5
	" "	1.2	19.6	38.4
	" "	2.0	18.0	39.6
3			1.4	4.0
	Adenosine triphosphate	6.0	11.3	24.4
	Yeast adenylic acid	6.0	1.5	
	Adenosine	7.5	1.7	3.8

be more susceptible than adenosine polyphosphate to the action of such enzymes, so that nothing definite can be said in regard to the optimum concentrations or the relative activities of mono- and polyphosphorylated adenine nucleotides until the α-ketoglutaric enzyme can be obtained in a purer condition. On the other hand, it is clear from the data of Table III that the action of the nucleotide is a catalytic one. Table III also shows that yeast adenylic acid and adenosine are inactive.

The optimum concentration of magnesium is about 0.002 M or 50 γ per cc.; manganese can replace magnesium but is less active (Table IV).

Diphosphothiamine—Attempts to split off diphosphothiamine in a revers-

ible manner have so far met with little success. After the enzyme suspensions are brought at 0° to pH 4.5 for 10 to 15 minutes with acetate buffer and the resulting precipitate is resuspended in phosphate buffer, a method successfully used by Green *et al.* (9) with their carboxylases, diphosphothiamine produced a small increase (25 to 45 per cent) of the rate of oxidation of α-ketoglutarate, while dialysis for 2 to 3 hours against pyrophosphate buffer at pH 9.5 and 0° did not cause any dissociation; both procedures led to marked inactivation of the enzyme.

Carriers—There is now much doubt regarding the participation of 4-carbon dicarboxylic acids as hydrogen carriers in biological oxidations (13), as originally postulated by Szent-Györgyi. Their effects can be explained by

TABLE IV

Effect of Magnesium and Manganese

The manometer bottles contained 1 5 cc. of enzyme dialyzed successively against 0.02 M pyrophosphate buffer of pH 8 5 and 0 05 M orthophosphate buffer of pH 7.5 each time for 2.5 hours, 0.003 M adenosine triphosphate, 0.025 M sodium malonate, and 0.02 M sodium α-ketoglutarate. The final volume was made up to 2 cc. with water. Temperature 36.5°. Gas phase 100 per cent oxygen. Incubation time 40 minutes.

Additions		Oxygen uptake	α-Ketoglutaric acid utilized
MgCl₂	MnCl₂		
micromoles	*micromoles*	*c.mm*	*mg.*
		98	1.63
2		307	
4		395	5.00
8		350	
	0.9	188	
	3.6	253	3.63

the fact that they are involved in condensations which are essential for the oxidation of pyruvic acid. It is clear from the data of Table V that 4-carbon dicarboxylic acids were not involved in the transport of hydrogen from α-ketoglutarate to oxygen, since the rate of oxidation of α-ketoglutarate was independent of the absence or presence of fumarate. Cozymase was added in all experiments of Table V in order to insure the functioning of the malate ⇌ oxalacetate (malic dehydrogenase) system; this was indicated by the fact that fumarate was readily oxidized by the enzyme suspensions. It might be argued that the succinate formed from α-ketoglutarate would overcome to some extent the malonate block of the succinic dehydrogenase so that eventually fumarate would become available to the system, but if fumarate were indispensable one would expect its addition to have a con-

siderable effect on the initial rate of α-ketoglutarate oxidation; that this was not the case is indicated (*cf.* Table V) by the values of the oxygen uptake during the first 10 minutes of incubation. An experiment with pyruvate (Experiment 2b, Table V) is given as a control of the extent to which 4-carbon dicarboxylic acids were absent from the enzyme preparations.

In contrast to the coenzyme dehydrogenases, the succinic dehydrogenase, and the pyruvic dehydrogenase of *Escherichia coli* (5) and brain (12), the

TABLE V

C4 Dicarboxylic Acids

The manometer bottles contained 1 5 cc. of dialyzed enzyme, 0.004 м $MgCl_2$, 0.03 м potassium phosphate buffer of pH 7 5, 0 003 м adenosine triphosphate, and 0 0007 м cozymase. The final volume was made up to 2 cc. with water. Temperature 36 5°. Gas space 100 per cent oxygen.

Experiment No	Additions (м × 10⁻³)				Time	Oxygen uptake	α-Ketoglutaric acid utilized	Pyruvic acid utilized
	Malonate	Fumarate	α-Keto-glutarate	Pyruvate				
					min	*c.mm*	*mg.*	*mg.*
1	25		35		10	63		
					40	233	3.17	
	25	2.2			10	16		
					40	38		
	25	2.2	35		10	80		
					40	258	3.12	
2a	50		20		10	29		
					40	82	1.73	
	50	2 2			10	14		
					40	36		
	50	2 2	20		10	37		
					40	99	1.34	
2b				14	10	27		
					40	70		0.31
		2.2			10	56		
					40	132		
		2.2		14	10	170		
					40	602		1.44

heart α-ketoglutaric dehydrogenase does not react or reacts only very slowly with artificial carriers such as methylene blue, pyocyanine, gallocyanine, thionine, and ferricyanide. The aerobic oxidation of α-ketoglutarate is inhibited by cyanide, suggesting a participation of the cytochrome system. Ferricytochrome *c* is rapidly reduced by α-ketoglutarate in the presence of the enzyme; the rate of reduction is identical with the rate of oxygen uptake, indicating that cytochrome *c* is a physiological carrier in the system. Thus in an experiment with 10 × 10⁻⁸ mole of ferricytochrome

c, 3×10^{-8} mole was reduced in 1 minute[3] at 24°, corresponding to an uptake of 0.17 c.mm. of O_2; in a parallel experiment at 24° with the same enzyme the rate of oxygen uptake was 6 c.mm. of O_2 per minute, which multiplied by the ratio of enzyme dilutions in the manometric and optical test respectively gives $6 \times 1.34/60 = 0.13$ c.mm. of O_2.

Fig. 3 shows that, under the conditions of the optical test, the reduction of cytochrome c is practically a linear function of time for the first 3 minutes, and that there is no reaction in the absence of either α-ketoglutarate or adenine nucleotide when dialyzed enzyme is used. Deficiency of inorganic
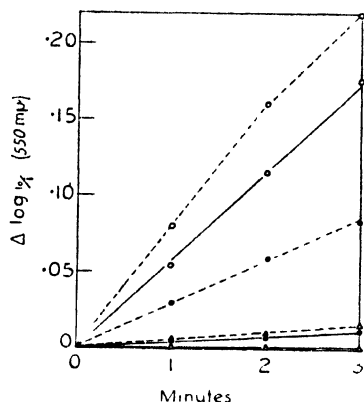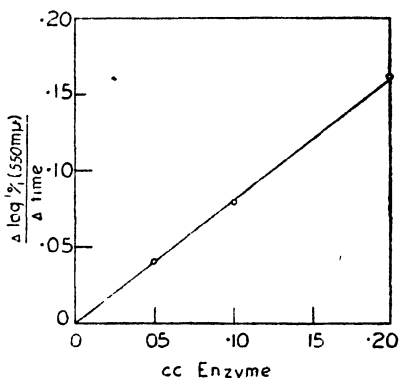

Fig 3. Fig 4.

FIG 3. Rate of reduction of cytochrome c The complete system contained 0 1 cc of enzyme, 0 0013 M $MgCl_2$, 0 025 M phosphate buffer (pH 7.3), 0 0001 M adenosine triphosphate, 0 05 M malonate, 12×10^{-8} mole of cytochrome c, 0 001 M KCN, and 0 003 M α-ketoglutarate. The final volume was made up to 6 cc. with water. Air; 24° Continuous lines, dialyzed enzyme; broken lines, undialyzed enzyme. O complete; ● no adenosine triphosphate; △ no α-ketoglutarate.

FIG 4 Rate of reduction of cytochrome c as a function of the enzyme concentration The conditions were the same as in the complete system of Fig. 3 with various amounts of undialyzed enzyme

phosphate or magnesium also gave suboptimal reaction rates. With undialyzed enzyme, probably because of its high dilution in this test, it was

[3] The concentration of ferricytochrome c (C_{ox}) was calculated by the equation

$$C_{ox} = \frac{1/d \log I_0/I - \alpha_{red} \times C_{total}}{\alpha_{ox} - \alpha_{red}}$$

where C_{total} = total cytochrome c concentration; α_{ox} = absorption coefficient of ferricytochrome c at 550 mμ = 0.0956 $\times$ 10^8 (cm ^{2} $\times$ moles^{-1}); α_{red} = absorption coefficient of ferrocytochrome c at 550 mμ = 0.281 $\times$ 10^8 (cm.^{2} $\times$ moles^{-1}); and d = length of light path in absorption cells = 1.306 cm.

necessary to add adenine nucleotide in order to obtain optimal reaction rates (Fig. 3). Fig. 4 shows that the rate of reduction of cytochrome *c* was proportional to the concentration of enzyme.

Properties of Enzyme—The substrate affinity of α-ketoglutaric dehydrogenase is high; under the conditions of the manometric test the maximum rate of oxygen uptake was reached with about 0.007 M α-ketoglutarate; the half speed concentration was about 0.0025 M (Fig. 5).

The pH-activity curve is shown in Fig. 6. The optimum pH is about 7.0; the enzyme activity falls off rather sharply to either side of the maximum. The reaction mixtures were buffered with 0.025 M phosphate and
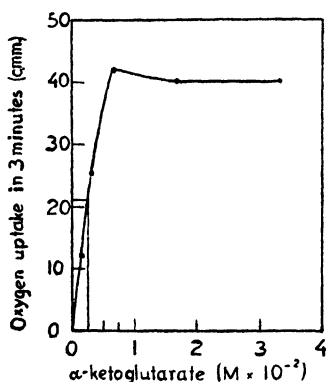
FIG. 5.   FIG. 6.

FIG. 5. Rate of oxygen uptake as a function of the concentration of α-ketoglutarate. The manometer bottles contained 1.5 cc. of enzyme, 0.004 M MgCl₂, 0.03 M phosphate buffer of pH 7 5, 0.0004 M adenosine triphosphate, and 0.05 M malonate. Final volume 2 cc ; oxygen; 36.5°.
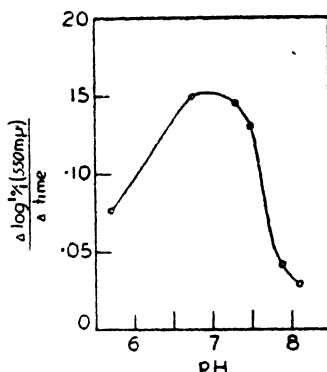
FIG. 6. Rate of reduction of cytochrome *c* as a function of pH. The conditions were the same as in the complete system of Fig. 3 with undialyzed enzyme.

the pH values were determined with the glass electrode after the spectrophotometric determinations had been made.

The α-ketoglutaric dehydrogenase is relatively stable in the enzyme suspensions that have been used in this work. When kept at 0° the activity decreases about 20 per cent in 24 hours and 50 to 60 per cent in 4 days. The enzyme withstands freezing relatively well (30 per cent loss of activity) but was completely destroyed by drying the suspensions in the frozen state. After centrifugation at 15,000 R.P.M. for 10 minutes at 10° about 90 per cent of the original activity was present in the sedimented particles and the remaining 10 per cent in the opalescent supernatant.

Preparations with α-ketoglutaric dehydrogenase activity were obtained from the heart of the cat, pig, and sheep; the relative activities were about

100, 50, and 35 respectively. Similar preparations from pigeon breast muscle were almost inactive. Pigeon brain homogenates (11) were fairly active (*cf.* Table I).

DISCUSSION

It is tempting to assume that there may exist a functional relationship between the dependence of the action of the animal α-ketoglutaric dehydrogenase upon the presence of adenine nucleotide and the large amount of phosphorylation caused by its activity. Succinyl phosphate, which may be a primary reaction product, is very rapidly dephosphorylated by the enzyme suspensions used in this work and by similar tissue preparations[4] in the absence of added adenine nucleotide, and it is difficult to visualize the function of the nucleotide unless it is assumed that it forms a dissociable compound with the dehydrogenase and that the transfer of phosphate between the phosphorylated primary dehydrogenation product and adenine nucleotide takes place on the surface of the dehydrogenase itself instead of being mediated by a second enzyme. It is conceivable that a large increase in the functional efficiency of this type of enzymes may have been achieved by incorporation of several prosthetic groups in the same protein or by formation of a highly specialized enzyme complex.

The present work gives no information as to the existence or otherwise of intermediate carriers between α-ketoglutarate and cytochrome c but it is likely that a flavine nucleotide (*cf.* (4, 14)) may be involved either as a prosthetic group of the dehydrogenase or as a separate flavoprotein.

EXPERIMENTAL

Preparation of Enzyme Suspension—The heart was chilled in ice and after removal of fat and connective tissue was finely minced and washed three times for 5 to 10 minutes at 0° with 15 to 20 volumes of 0.9 per cent potassium chloride with brisk mechanical stirring, the tissue being separated each time by centrifugation. The last washing was almost colorless. The washed mince was ground at 0° with sand and 2 volumes of either 0.9 per cent potassium chloride or 0.065 M potassium phosphate buffer of pH 7.5 and the resulting paste was centrifuged at low speed for 2 to 3 minutes. The supernatant was kept at 0° until used. There was a fairly wide range of variation in the activity of individual preparations.

Dialysis—When desired the enzyme suspensions were dialyzed for 5 hours in thin collodion tubes (about 1.3 cm. in diameter) against 3.5 liters of either 0.4 per cent potassium chloride or 0.05 M potassium phosphate buffer of pH

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[4] Lipmann, F., personal communication; Ochoa, S., unpublished. The same is true of acetyl phosphate. I am very indebted to Dr. Lipmann for samples of succinyl phosphate and acetyl phosphate.

7.5 at 0.5–2° unless otherwise stated.   Continuous mechanical rocking of the dialysis tubes and outside stirring insured rapid diffusion.   There always occurred some irreversible inactivation of the enzyme when the dialysis time exceeded 2 hours and it increased with increasing duration of dialysis.

Manometric Test—The main space of the Warburg bottles contained 1.5 cc. of enzyme plus various additions as indicated and the final volume was made up to 2.0 cc. with water; the center well contained alkali.   0.1 cc. of a freshly prepared neutralized solution of α-ketoglutaric acid was placed in the side bulb and was mixed with the enzyme just before the bottles were placed in the thermostat.   5 minutes were allowed for temperature equilibration.   For extrapolation of the oxygen consumption back to zero time it was assumed that the oxygen uptake during temperature equilibration was 70 per cent of that occurring during the first 5 minutes of measuring the gas exchange.   Respiratory quotients were determined as in previous work (8).   Anaerobic CO_2 evolution was determined as the sum of CO_2 evolved during incubation plus that liberated at the end by acidification of the reaction mixtures.

Optical Test—0.1 cc. of enzyme with additions as indicated in Fig. 3 was made up with water to 6 cc.   Additions were made in the following order: magnesium chloride, adenine nucleotide, malonate, water, phosphate buffer, cytochrome c (10 to 12 × 10^{-8} mole), enzyme, cyanide, and α-ketoglutarate. 2 minutes were allowed for temperature equilibration after addition of the ice-cold enzyme; 0.06 cc. of neutralized 0.1 M potassium cyanide was next added and the extinction of light of the wave-length 550 mμ was measured (zero time).   0.05 cc. of α-ketoglutarate was then added and the extinction values were measured at 1 minute intervals thereafter.   The test was carried out in air at room temperature (24–25°).   Cyanide was added in order to prevent reoxidation of ferrocytochrome c by the cytochrome oxidase; as used in the test it does not interfere with the reduction of cytochrome c (*cf.* (15)).   The light extinction was measured with a Coleman type 10-S photoelectric spectrophotometer with an exit slit of 5 mμ.   The width of the absorption cells was $d = 1.306$ cm.

Analytical Methods—α-Ketoglutaric and pyruvic acids were determined according to Friedemann and Haugen (16) in trichloroacetic acid filtrates. Succinic semialdehyde was determined in a similar manner as the 2,4-dinitrophenylhydrazone.   The fact that the color given by the latter on addition of alkali fades very rapidly, whereas that given by the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid is very stable was utilized in determining α-ketoglutaric acid in the presence of succinic semialdehyde. Phosphate was determined as in previous work (8).   Succinic acid was determined manometrically with succinoxidase according to Krebs and Eggleston (17).   The method gives the sum of succinate plus α-ketogluta-

rate present and the amount of preformed succinate was obtained by subtracting the succinate equivalent of the α-ketoglutarate found by the hydrazone method. All estimations were carried out in duplicate and agreed to within 5 per cent.

Chemical Preparations—α-Ketoglutaric acid was prepared according to Neuberg and Ringer (18) and recrystallized from ethyl acetate with petroleum ether as described by Wislicenus and Waldmüller (19); it melted at 116–117°. Succinic semialdehyde was prepared by the method of Carriere (20); the 2,4-dinitrophenylhydrazone (recrystallized from 95 per cent alcohol) melted at 204°. Freshly distilled succinic semialdehyde was diluted with water and neutralized with the calculated amount of sodium hydroxide. Cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (21) and its concentration was determined spectrophotometrically with the absorption coefficients given by Theorell (22). Adenosine triphosphate and muscle adenylic acid were prepared as previously described (8).

SUMMARY

Cell-free suspensions of washed heart muscle contain a very active α-ketoglutaric dehydrogenase. When the succinic dehydrogenase is inhibited by malonate, and in presence of glucose, the preparations catalyze the oxidative decarboxylation of α-ketoglutaric acid to succinic acid and carbon dioxide, while 3 molecules of phosphate are simultaneously esterified to the sugar forming hexose diphosphate. The oxidative decarboxylation of α-ketoglutarate is not caused by the action of a carboxylase followed by that of an aldehyde oxidase but by a single enzyme or enzyme complex. Inorganic phosphate, magnesium ions, and either muscle adenylic acid or adenosine triphosphate are required for activity of the α-ketoglutaric dehydrogenase. Arsenate cannot be substituted for phosphate. Cytochrome *c* is a physiological electron carrier between α-ketoglutarate and molecular oxygen. The system of 4-carbon dicarboxylic acids is not involved in the hydrogen transfer.

BIBLIOGRAPHY

1. Breusch, F. L., *Science* **97**, 490 (1943). Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).
2. Weil-Malherbe, H., *Biochem. J.*, **31**, 299 (1937).
3. Barron, E. S. G., and Miller, C. P., Jr., *J. Biol. Chem.*, **97**, 691 (1932).
4. Lipmann, F., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 248 (1939).
5. Still, J. L., *Biochem. J.*, **35**, 380 (1941).
6. Ochoa, S., *J. Biol. Chem.*, **149**, 577 (1943).
7. Barron, E. S. G., Goldinger, J. M., Lipton, M. A., and Lyman, C. M., *J. Biol. Chem.*, **141**, 975 (1941).
8. Ochoa, S., *J. Biol. Chem.*, **151**, 493 (1943).
9. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69 (1942).
10. Weil-Malherbe, H., *Biochem. J.*, **31**, 2202 (1937).
11. Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, **33**, 1980 (1939).
12. Long, C., *Biochem. J.*, **37**, 215 (1943).
13. Potter, V. R., *J. Biol. Chem.*, **134**, 417 (1940). Straub, F. B , *Enzymologia*, **9**, 148 (1940).
14. Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940)
15. Lockhart, E. E., and Potter, V. R., *J. Biol. Chem* , **137**, 1 (1941).
16. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem* , **147**, 415 (1943)
17. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 442 (1940)
18. Neuberg, C., and Ringer, M., *Biochem. Z.*, **71**, 228 (1915).
19. Wislicenus, W., and Waldmuller, M., *Ber. chem Ges* , **44**, 1566 (1911).
20. Carriere, E., *Ann chim.*, **17**, 41 (1922).
21. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937)
22. Theorell, H., *Biochem Z.*, **285**, 207 (1936)

A DIFFERENCE IN THE METABOLISM OF *l*- AND *dl*-TRYPTO-PHANE IN THE HUMAN*

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While studying the rates of tryptophane excretion in humans subsequent to the ingestion of 2 gm. of *l*- or *dl*-tryptophane (Merck), we observed a deep purple coloration on the addition of the Hopkins-Cole precipitating reagent (requisite for the tryptophane determination) to the urine specimens collected from the first subject to whom *dl*-tryptophane had been fed. Although we had already performed numerous tryptophane determinations on urine of normal subjects who had ingested *l*-tryptophane and of subjects on tryptophane-deficient diets, this phenomenon had not been previously observed. To test the possibility of this being an individual or transient characteristic, the experiment was repeated and the finding confirmed in the same individual after a lapse of several days, as well as in another subject. It appeared, therefore, that this pigment formation is a characteristic effect due to the ingestion of the *d* component of *dl*-tryptophane by the human adult.

Application of a number of qualitative color reactions to these abnormal urine specimens indicated that the aberrant substance might be an indole derivative. A heavy reddish purple precipitate is formed on the addition of 0.1 N iodine solution to the urine of subjects who have received *dl*-tryptophane, but no precipitation takes place after the ingestion of *l*-tryptophane. The properties of the product are similar to those of indigo red. However, although a number of properties of the *d*-tryptophane metabolite which gives rise to indigo red in the presence of iodine have been observed, its isolation has not been achieved nor its identity established by reason of its close similarity to other indole compounds in the urine. Indeed, its only true differentiating characteristic lies in the formation of indigo red in the presence of iodine in neutral or slightly acid solution.

EXPERIMENTAL

Human Experiment—Following our first observations an effort was made to determine quantitatively the amount of aberrant metabolite by means

of the iodine reaction.   For this purpose four normal adults were fed 1 gm.
of *l*-tryptophane (Merck) with 300 cc. of water 2 hours after a standard light
breakfast and the following day 1 gm. of *dl*-tryptophane (Merck) was simi-
larly given.   The urine was collected at hourly intervals and the volume
recorded.   The output of the metabolite was determined by the following
quantitative adaptation of the iodine test mentioned below. _ To 8 cc. of
filtered urine in weighed 15 cc. centrifuge tubes were added 2 cc. of 0.1 N
iodine solution and the reaction mixture was refrigerated overnight.   After
centrifugation, the supernatant fluid was decanted and discarded.   The
precipitates were then washed three times by resuspension in 10 cc. of dis-

TABLE I

*Excretion of Unknown Indole Derivative after Ingestion of 1 Gm. of dl- and l-Tryptophane
by Normal Adult Humans*

The results listed are for the *dl* form; no indigo red formed in the urine of sub-
jects fed *l*-tryptophane.

Time after ingestion	Unknown indole derivative as indigo red*			
	Subject W, female, 53 6 kilos	Subject I, female, 62.0 kilos	Subject H, male, 75 0 kilos	Subject B, female, 50 0 kilos
hrs	*mg*	*mg*	*mg*	*mg.*
0	0	0	0	0
1	56.50	18 75	60.60	72.30
2	41 80	23.00	62.50	41.30
3	23.45	81 60	70 20	39.60
5	30 80	145.50	121.10	115.50
6	5.00†	0	0	0
Total excretion	157 55	268 85	314 40	268.70

* By calculation, 1 gm. of *dl*-tryptophane $\approx$ 0 5 gm. of *d*-tryptophane $\approx$ 322 mg.
of indigo red.
† 0 excretion at the 7th hour.

tilled water, centrifugation, and decantation of the supernatant liquid.
The final residues in the tubes were dried to constant weight in a 60° oven
and the weight of the insoluble product determined by difference.   The
data from these experiments are shown in Table I.   It will be observed that
no indigo red formed in the urine of the subjects when *l*-tryptophane was
fed, but that considerable amounts were obtained when these same subjects
received *dl*-tryptophane.
    To date this biological difference of *l*- and *dl*-tryptophane has been tested
by thirty-four experiments on humans and in every instance the results
have confirmed the original observations.   These experiments have
brought forth the following additional information of physiological interest:

(a) elimination of the metabolite is not affected by the intake of food before or after ingestion of racemic tryptophane; (b) its excretion usually subsided 6 to 7 hours after administration of 1 gm. of dl-tryptophane and 8 to 9 hours after 2 gm. of the amino acid had been fed; (c) in general the output rises to a maximum and then falls off sharply to zero.

Isolation and Identification of Indigo Red Formed by Action of Iodine on Urines of Subjects Fed dl-Tryptophane—Each of six normal adult humans was given 1 gm. of dl-tryptophane (Merck) by mouth with 300 cc. of water and the urine specimens were collected at hourly intervals to the 4th hour after administration of the amino acid. Each specimen was tested by adding 2 cc. of 0.1 N iodine solution to 8 cc. of urine; on being cooled in the ice box for 1 to 2 hours all the samples contained the characteristic reddish purple precipitate. The specimens were pooled and filtered by gravity through fluted paper; a total of 950 cc. of urine was obtained. To 500 cc. of this urine were added 100 cc. of iodine solution and the mixture was refrigerated overnight at 4°. Experience having shown filtration by gravity or suction to be an unsatisfactory separation procedure, the precipitate was collected in a weighed 50 cc. cone bottom tube by centrifugation and washed five times by resuspension in distilled water, centrifugation, and careful decantation of the rose-tinted supernatant solution. On being dried to constant weight in a 60° oven, the washed product was found to weigh 153.5 mg. and contained 10.85 per cent N by micro-Kjeldahl analysis (1). On recrystallization of 50 mg. of this crude substance from methyl alcohol, 34 mg. of a purple plate-shaped crystalline material were obtained which contained 10.61 per cent N. A second recrystallization did not change the nitrogen value significantly, although an over-all manipulative loss of 24 mg. was incurred. When a small sample of the product was heated in an open flame, a characteristic indole odor was obtained. The sublimation range of the substance was found to be 300–340°, which coincides with that reported for indigo red, 295–340° (2). The nitrogen (1) and carbon (3) analyses are in agreement with the formula for indigo red or blue.

$C_{16}H_{10}N_2O_2$ Calculated, C 73.20, N 10 69; found, C 73 01, N 10.61

The probable identity of the isolated product with indigo red was deduced from the following evidence. The compound is the result of an oxidative process, since the consumed iodine could be quantitatively recovered by thiosulfate titration on treatment of an aliquot of the filtrate of the reaction mixture with potassium iodate. This was further demonstrated by the fact that bromine water, hydrogen peroxide, and sodium hypochlorite in acid solution all yield dark purple pigments on addition to the urine derived from subjects fed dl-tryptophane. The literature (2) indicated that indigo blue and indigo red are usual products resulting from the action of these oxidiz-

ing agents on the urine.   When an alkaline solution of the isolated product with glucose or sodium hydrosulfite was warmed, the original deep red color of the solution disappeared.   An acetone solution of the isolated product exhibited a maximum absorption in the region 540 to 560 mμ with a peak which is similar to that reported for indigo red (4).   An authentic sample of indigo blue in acetone was found to have a maximum absorption in the range 580 to 600 mμ and a peak at 600 mμ.   This suggests the identity of the unknown product to indigo red rather than indigo blue.

The compound is insoluble in cold or hot water and 10 per cent hydrochloric, sulfuric, or nitric acid.   It dissolves in concentrated sulfuric acid to form a yellowish green solution which becomes deep brown on standing. The compound dissolves readily in 10 per cent sodium hydroxide or potassium hydroxide, from which it is readily precipitated on neutralization with hydrochloric acid.   Excessive heating of the alkaline solution results in some decomposition of the product.   Solution could not be effected even in 50 per cent potassium carbonate.   It is readily soluble in hot methyl or ethyl alcohol or acetone and slightly soluble in chloroform or ether.   On standing overnight these solutions take on a definite bluish tint.   Solution in glacial acetic acid is easily effected but on heating the color changes to a greenish yellow tint.   A comparison of these solubility data with those reported for indigo red (2) lends validity to the view that the isolated product is indigo red.

Survey of Possible Metabolites of Tryptophane Giving Rise to Indigo Red

Inasmuch as numerous attempts at isolation and identification of the dl-tryptophane metabolite which gives rise to indigo red have to date been futile, a study of some probable tryptophane metabolites was made to determine which of these reacted similarly to the unknown substance.

Tryptophane—The addition of iodine solution to normal urine to which dl-tryptophane (Merck) has been added results in the formation of a tan-brown precipitate on refrigerating overnight.   This dissolves readily in acetone to a chocolate-brown solution and contains 6.14 per cent N; the calculated N for diiodotryptophane is 6.11 per cent.   Clearly, this compound is not similar to the isolated product.   Moreover, the augmentation of the tryptophane output of the subjects was about the same on administration of l- or dl-tryptophane.

Tryptamine—Iodine solution was found to react with tryptamine in normal urine, giving an insoluble brown product which is very soluble in acetone and contains 6.78 per cent N; the theory for diiodotryptamine is 6.81 per cent N.   This substance also cannot be regarded as the immediate precursor of indigo red.

Indoleacetic and Indolepropionic Acids—Neither of these two compounds

formed insoluble substances on treatment with iodine solution under the conditions of the test and they cannot therefore be regarded as immediate sources of indigo red in the urine.

Indoxyl (Indican)—Although indoxyl would be expected to be the source of indigo red obtained from the urine of humans fed *dl*-tryptophane, the data in Table II indicate that this is not so. It is obvious that the amount of indican present as determined by the method of Sharlit (5) could in no way account for the amount of indigo red isolated from the urine of the subjects given *dl*-tryptophane or the failure to obtain indigo red subsequent to *l*-tryptophane administration. These considerations preclude the possibility of indoxyl acting as a source of indigo red in this instance.

TABLE II

Excretion of Indole Substances after Ingestion of 2 Gm. of l- and dl-Tryptophane by Normal Adult Humans

Subject, sex, and weight	Time after inges-tion	Excretion of indole substances*			Subject, sex, and weight	Time after inges-tion	Excretion of indole substances*		
		l-Tryp-to-phane†	*dl*-Tryptophane				*l*-Tryp-to-phane†	*dl*-Tryptophane	
		Indican	Indican	Indigo red			Indican	Indican	Indigo red
	hrs	*mg*	*mg*	*mg*		*hrs*	*mg.*	*mg.*	*mg.*
Subject A,	0.0	0 71	1.17	0 0	Subject F,	0.0	1.20	1.59	0
male, 70	0.5	0 70	0.95	4.3	female,	0.5	0.41	0.42	0
kilos	1.0	0.39	0.98	38.1	60 kilos	1.0	0.42	0.42	0
	2.0	0.49	2.34	20.5		2.0	0.63	0.68	18.3
	3.0	0.45	1.80	51.4		3.0	0.57	1.33	16.8
	4.0	0.40	0.46	62.4		4.0	0.50	0.62	70.0
	9.0			0		8.0			0

* The iodine test was applied qualitatively after the 4th hour.
† All readings for indigo red were 0.

Skatole and Indole—These two substances were excluded as indigo red sources by the finding that, on distillation of urine samples collected from subjects fed *dl*-tryptophane under the conditions described by Zoller (6) as optimal for the volatilization of indole or skatole, the distillate failed to yield indigo red on treatment with iodine solution and an aliquot of the residue yielded the same amount of indigo red on addition of iodine solution as was obtained from a comparable amount of the original sample. In subject A, male, given 1 gm. of *dl*-tryptophane, the 3rd hour specimen measured 36 cc. and yielded 1.95 mg. per cc. of indigo red by the iodine reaction. The reaction of 25 cc. of this urine was made alkaline to phenolphthalein with 10 per cent NaOH and concentrated to 10 cc. by distillation. The

residue was then made neutral to litmus with dilute HCl and the volume readjusted to 25 cc. When 8 cc. of this solution were submitted to the iodine reaction, 1.90 mg. per cc. of indigo red were obtained. Moreover, the distillate failed to give a positive iodine reaction for indigo red, a Salkowski nitrosoindole (7) reaction for indole, or Herter's (8) p-dimethyl-aminobenzaldehyde blue reaction for skatole. Inasmuch as *indole-3-alde-hyde* and *indole-3-carboxylic* acid could be expected to be degraded to indole (9) under the condition of this distillation procedure, these two substances are also precluded from consideration as the precursor of indigo red.

Although it is evident from the foregoing data that the *dl*-tryptophane metabolite which gives rise to indigo red on treatment with iodine solution is an indole derivative, it is clearly not one of the substances commonly regarded as a product of intermediary metabolism of tryptophane (10). It has been found to be stable in the urine on heating for 1 hour in the presence of 10 per cent sodium hydroxide or sulfuric acid. The metabolite could not be extracted from neutral or acidified urine with chloroform, ether, or iso-butyl alcohol. Direct application of the nitroso reaction to these urines yielded an orange-red color which could be extracted with isobutyl alcohol but not chloroform or ether. From the literature (2), this is suggestive of the absence of indole and the possible presence of indoleacetic acid, which has been ruled out by previous experiment. On treatment of a sample of this urine with Obermayer's reagent (11) a deep red color formed which could not be extracted with chloroform or ether but readily extracted with isobutyl alcohol. The failure to obtain a blue color with this reagent de-notes the absence of appreciable amounts of indican and the solubility of the red color in isobutyl alcohol according to Salkowski (12) suggests the pres-ence of indoleacetic acid. It is to be noted, however, that although these color reactions were intense in the urine of subjects fed *dl*-tryptophane, they were also obtained in a lesser degree with normal urine or urine of subjects fed *l*-tryptophane. Obviously, the unspecific nature of these color reac-tions does not permit clear interpretation of the data so obtained.

In this survey no direct tests for other possible intermediary metabolites of tryptophane which might give rise to indigo red have been made. How-ever, some of these are precluded to a certain extent by circumstantial evidence. Although *indoxylic acid* is known to be oxidized readily to indigoid pigments, the non-volatile nature of the unknown substance obviates its possible identity with this acid. The failure of the product to dissolve in ether, chloroform, or isobutyl alcohol on extraction in a measure excludes its probable identity with *indolelactic acid, indolepyruvic acid* (13), and a number of other possible indole compounds. The fact that a deep red and not a blue color was obtained when the urine of the subjects who had received *dl*-tryptophane was treated with Ehrlich's aldehyde reagent

points to the presence of increased amounts of indole rather than skatole derivatives (8). However, this color reaction cannot be regarded as sufficient evidence for the exclusion of a skatole derivative as the possible intermediate.

Comment

Although the d and l forms of tryptophane have been reported by Berg (14) and du Vigneaud, Sealock, and Van Etten (15) to be equally effective in promoting growth in rats, there is evidence that mice fail to grow (16) or grow poorly (17) on diets supplemented with d-tryptophane. Our present studies indicate that the metabolism of the d component of dl-tryptophane in the human differs markedly from that of l-tryptophane. From the data in Tables I and II it appears that the greater part of the metabolized d component can be recovered as indigo red when the urine is treated with iodine solution within 4 to 5 hours after ingestion. Although the intermediate metabolite has not been isolated, it is clearly not utilizable by the human. The practical implication of this finding to human nutritional studies would appear to be that nearly double quantities of the racemic tryptophane need to be administered to secure the equivalent effect of a calculated amount of the naturally occurring variety.

SUMMARY

It has been observed that the metabolism of the d component of dl-tryptophane differs from that of the l component in the human. Although the aberrant intermediate metabolite has not been isolated, some of its properties have been reported. Its most characteristic reaction is the formation of indigo red on the addition of iodine. The study suggests that the biological value of dl-tryptophane is approximately one-half that of l-tryptophane.

BIBLIOGRAPHY

1. Meeker, E. W., and Wagner, E. C , Ind and Eng. Chem , Anal. Ed., 5, 396 (1933).
2. Ellinger, A , in Neubauer, C., and Huppert, C H., Analyse des Harns, Wiesbaden, 11th edition, 2, 802–806, 865, 883 (1913).
3. Nicloux, M., Bull. Soc chim. biol , 9, 639 (1927); Compt. rend. Acad , 184, 890 (1927); Compt. rend Soc biol., 98, 1222 (1928). Boivin, A., Bull. Soc. chim. biol., 11, 1269 (1929)
4. Schultz, G , Farbstofftabellen, Leipzig, 7th edition, 1, 600 (1931).
5. Sharlit, H., J. Biol Chem , 99, 537 (1932–33).
6. Zoller, H F., J. Biol. Chem., 41, 25, 37 (1920).
7. Salkowski, E , Z. physiol Chem., 8, 425 (1883–84).
8. Herter, C. A., J. Biol. Chem., 4, 239, 253 (1908). Herter, C. A., and Foster, M. L., J. Biol. Chem., 2, 267 (1906–07).
9. Zemplén, G., in Abderhalden, E , Biochemisches Handlexikon, Berlin, 4, 906, 902 (1911).

10. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Interpretations, Baltimore, 513 (1931).
11. Obermayer, F., *Wien. klin. Wochschr.*, 9, 176 (1890). Also see Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 929 (1937).
12. Salkowski, E., *Z. physiol. Chem.*, 9, 23 (1885).
13. Bauguess, L. C., and Berg, C. P., *J. Biol. Chem.*, 104, 675 (1934).
14. Berg, C. P., and Potgieter, M., *J. Biol. Chem.*, 94, 661 (1931–32). Berg, C. P., *J. Biol. Chem.*, 104, 373 (1934).
15. du Vigneaud, V., Sealock, R. R., and Van Etten, C., *J. Biol. Chem.*, 98, 565 (1932).
16. Kotake, Y., Ichihara, K., and Nakata, H., *Z. physiol. Chem.*, 243, 253 (1936).
17. Totter, J. R., and Berg, C. P., *J. Biol. Chem.*, 127, 375 (1939).

CHEMICAL DETERMINATION OF PYRIDOXINE.
REACTIONS IN PURE SYSTEMS

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Critical studies have been conducted in our laboratories on various methods for the determination of pyridoxine. Our experiences with the microbiological method have been presented elsewhere (14). The present paper is concerned with the chemical determination of pyridoxine based upon its coupling reaction with 2,6-dichloroquinonechloroimide. For a better understanding of the factors modifying the reaction and how these should be controlled, tests were first conducted with pure systems. The results of these studies are now presented. In the following paper (5) the application of the method to pharmaceutical preparations and biological materials is described.

The phenolic nature of pyridoxine has been the basis for chemical determination through coupling reactions with 2,6-dichloroquinonechloroimide (15, 11), diazotized sulfanilic acid (16, 1), the Folin-Denis reagent (16), and ferric chloride. However, even the chloroimide reagent, the most specific of these, reacts to form dyes with many other phenols, amines, and unrelated compounds (11). The reliability of each of the chemical methods for pyridoxine has been largely dependent upon the removal from the test extract of such interfering substances.

Principle of Present Method

The vitamin is coupled with 2,6-dichloroquinonechloroimide in a strongly buffered alcoholic solution to yield a blue pigment, which is estimated

(1) Pyridoxine   2,6-Dichloroquinone-
chloroimide

Blue pigment

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photometrically. The reaction between the vitamin and the reagent is believed to occur according to Equation 1 (4,12). Potential errors due to other compounds coupling with the reagent are eliminated by conducting the reaction concurrently in the presence of an excess of borate, which under proper conditions renders the pyridoxine non-reactive without affecting the reaction of other coupling compounds. The structural formula of the pyridoxine-borate complex is believed (12) to be

(2)

$$\left[\begin{array}{c} CH_2-O \quad O-CH_2 \\ HOCH_2 \underset{N}{\overset{}{\bigcirc}} -O \overset{B}{\diagdown} O- \underset{H_3C \diagdown N}{\overset{}{\bigcirc}} CH_2OH \\ CH_3 \quad H_3C \end{array} \right]^{-}$$
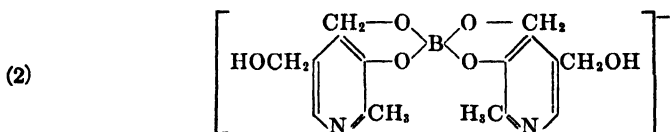
Reagents—

*Chloroimide Reagent—*100 mg. of recrystallized 2,6-dichloroquinone-chloroimide are dissolved in 250 cc. of isopropanol.[1] The solution is stored in a glass-stoppered bottle in the refrigerator, and portions are withdrawn as needed. The reagent should not be kept for more than 1 month, and should be discarded sooner if a pink discoloration develops. The solid reagent is first purified by dissolving 1 gm. in 50 cc. of acetone and precipitating by the gradual addition of small amounts of water while stirring. The crystals are collected on a Buchner funnel, rapidly air-dried by suction, and then stored in a sealed bottle in the refrigerator. The recrystallized reagent is stable for more than 6 months under such conditions of storage.

*Ammonia-Ammonium Chloride Solution—*160 gm. of ammonium chloride, C.P., are dissolved in 700 cc. of water and 160 cc. of concentrated ammonia water (approximately 27 per cent) added. The solution is diluted with water to 1 liter.

*Boric Acid Solution—*5.0 gm. of powdered boric acid, C.P., are dissolved in 100 cc. of distilled water.

*Pyridoxine Hydrochloride[2]—*100 mg. of the crystalline vitamin are dissolved in 1000 cc. of 0.1 N hydrochloric acid. This stock solution is stable for at least 3 months if stored in the refrigerator in an amber bottle. Working standards are prepared daily by dilution of the stock solution.

*Color Development—*A direct reading photoelectric colorimeter is most satisfactory for the measurements.[3] To a tube containing 1 cc. of pyridoxine solution, 5 cc. of isopropanol, 2 cc. of ammonia-ammonium chloride

[1] The crystalline compound may be prepared by the method described by Gibbs (4) or purchased from the Eastman Kodak Company, Chemical Sales Division, Rochester, New York, or from the Organic Products Company, New York.

[2] Obtained from Merck and Company, Inc , Rahway, New Jersey. In the present study, the terms pyridoxine and pyridoxine hydrochloride are used interchangeably, and all values are expressed as the hydrochloride.

[3] The Evelyn photoelectric colorimeter obtained from the Rubicon Company, Philadelphia, was employed in these studies.

solution, and 1 cc. of boric acid solution, 1 cc. of the chloroimide reagent is added, and the instrument is set at 100 per cent transmission 60 seconds later.[4]   The color is developed in 60 seconds in a similar tube containing water in place of the boric acid solution.   Readings are taken with the 620 mμ filter.

Though pyridoxine is destroyed by irradiation, especially in alkaline solution (6, 7), exposure to ordinary laboratory light for 3 hours produces no measureable destruction of the vitamin even in colorless solutions.   Only in the case of the standard solution of pyridoxine, which may be used
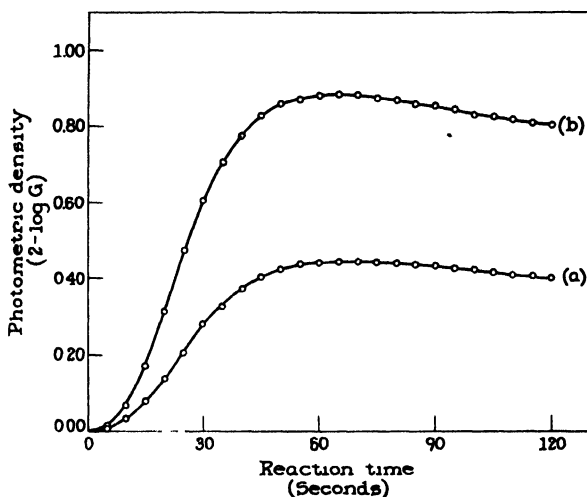

FIG 1 Rate of formation and fading of the dye formed in the reaction between pyridoxine and 2,6-dichloroquinonechloroimide.   Curve a, 10 γ of pyridoxine per tube; Curve b, 20 γ of pyridoxine per tube.

repeatedly for months, should special precautions be taken to avoid light destruction.

Typical time reaction curves showing the rate of formation of the colored complex are presented in Fig. 1.   The curves for 10 and 20 γ of pyridoxine were corrected for the small, progressive changes in the reagent blank before being plotted.   The graph demonstrates that the dye forms rapidly during the 1st minute and is sufficiently stable at 60 seconds for precise measurement.   When a veronal buffer (11) is used in the same water-isopropanol system, 20 minutes are required for maximal color development. In the water-butanol system of Scudi (11) the coupling reaction is completed only after 40 minutes.

[4] The solutions are added in the order mentioned to prevent the precipitation of salts.

In Fig. 2 are plotted data indicating the proportionality of the photometric density of the pigment to initial concentration of pyridoxine, exactly 60 seconds after the addition of chloroimide reagent. It is apparent that the reaction obeys Beer's law. When readings are taken at any time other than in the stage of maximal color development, deviations from the linear relationship between color and pyridoxine content may be found.

Reproducibility and Specificity of Method—More than 200 measurements over a period of a year on pure solutions containing 10 and 20 γ of pyridoxine per tube, with different sets of buffer and reagent solutions, some freshly prepared, others 2 to 3 weeks old, have given values agreeing with those plotted in Fig 2 to within ±1.1 per cent average deviation. In all
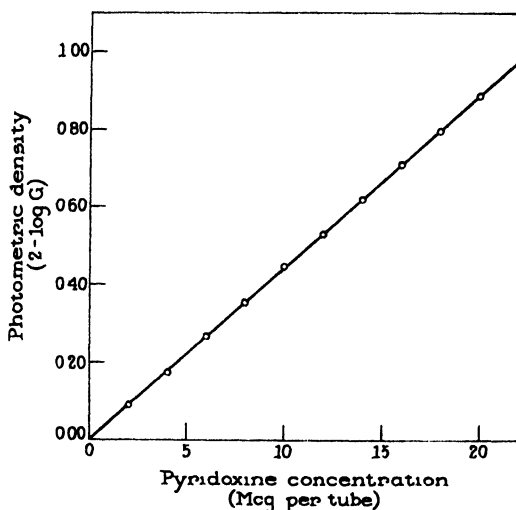

FIG. 2. Proportionality of photometric density of pigment to initial concentration of pyridoxine.

cases, the same batch of recrystallized 2,6-dichloroquinonechloroimide was employed for making the reagent.

Experimental evidence has been obtained proving the specificity of the method for pyridoxine. The vitamin reacts to form a dye with 2,6-dichloroquinonechloroimide but fails to do so in the presence of boric acid. Other compounds which couple with the reagent react to the same degree in the presence or absence of the boric acid. The photometric densities of the dyes due to pyridoxine and to interfering compounds are additive. These points are clearly illustrated in Table I.

Only the diacetyl derivative of pyridoxine, 2-methyl-3-hydroxy-4, 5-bis-(acetoxymethyl)pyridine showed an appreciable difference in reaction in

the presence and absence of boric acid. The compound is reputed to be biologically equivalent to pyridoxine on a molar basis, owing to the ease of removal of the acetyl groups from the molecule (17). The sample was assayed microbiologically (14) in our laboratory and found to possess 30 per

TABLE I

Validity of Borate Blank in Determination of Pyridoxine in Presence of Other Compounds Reacting with 2,6-Dichloroquinonechloroimide Reagent

| Compound | Amount* per tube | Photometric density† at 620 mμ | | | | | Inactivation of increment by boric acid |
| | | Test | (a) + boric acid | (a) + 10 γ of pyridoxine | (a) + 10 γ of pyridoxine and boric acid | Increment due to 10 γ of pyridoxine, (c) − (a) | |
		(a)	(b)	(c)	(d)	(e)	
	γ						*per cent*
Aniline	10,000	0.208	0.208	0.645	0.206	0.437	100
Phenol	500	0.220	0.220	0.658	0.226	0.438	99
β-Naphthol	50	0.241	0.241	0.680	0.240	0.439	100
Naphthylamine hydrochloride	50	0.145	0.146	0.586	0.146	0.441	100
Catechol	25	0.289	0.290	0.737	0.281	0.448	100
Resorcinol	25	0.103	0.103	0.549	0.112	0.446	98
3-Hydroxypyridine	4.6	0.006	0.006	0.450	0.018	0.444	97
2,4-Dimethyl-3-hydroxy-5-hydroxymethylpyridine hydrochloride	8.4	0.352	0.334	0.775	0.344	0.423	98
2-Methyl-3-hydroxy-4,5-bis(acetoxymethyl)pyridine	12.3	0.420	0.299	0.868	0.310	0.448	98
2-Methyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine dihydrochloride monohydrate	13.9	0.000	0.001	0.449	0.014	0.449	97
2-Methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine	8.9	0.440	0.432	0.875	0.445	0.435	97

* The weights of the pyridoxine analogues taken are equivalent on a molar basis to 10 γ of pyridoxine. These compounds were kindly supplied by Dr. K. Folkers of Merck and Company, Inc., Rahway, New Jersey.

† The colorimeter was set at 100 per cent transmission with the reagent blank.

cent of the potency of pyridoxine on a molar basis, a value in very close agreement with that derived from the chemical test. This may have been due to the presence of free pyridoxine in the sample. The acid-hydrolyzed material gave much higher values, equivalent to 75 to 85 per cent of pyridoxine when subjected to both microbiological and chemical assay. The

other analogues of pyridoxine had no appreciable stimulatory action on the yeast, *Saccharomyces cerevisiae*, before or after acid hydrolysis (5). They likewise failed to show any significant differences in reaction with the reagent in the two buffers. Biological assays on the rat have shown these compounds to possess practically no vitamin B_6 activity (17).

Böeseken (3) reported that boric acid forms complexes with 1,2-dihydroxy compounds. Scudi (11) found this to be true also in the case of pyridoxine. In natural extracts other compounds may compete for the boric acid. However, under the conditions of our test, there is always an excess of borate present for complete inactivation of the pyridoxine (5). Thus, in routine assays of a large variety of biological materials, it has been found in every case that added pyridoxine was rendered more than 97 per cent non-reactive in the presence of borate.

As a result of the tests recorded in Table I, it has been considered expedient in testing biological materials to set the instrument at 100 per cent transmission with a reaction tube containing the test extract and boric acid, 60 seconds after the addition of the reagent. The pigment formed in testing the same extract in the absence of borate is then determined photometrically. By this procedure only the additional light absorption due to the dye formed by the pyridoxine is measured.

Increment Procedure for Evaluating Photometric Density of Dye Formed by Pyridoxine—In the presence of relatively high concentrations of some interfering coupling compounds, the reaction between pyridoxine and 2,6-dichloroquinonechloroimide may be partially or completely inhibited within the period of measurement.

Illustrative data with two such interfering compounds are presented in Table II. In the presence of sufficient resorcinol or naphthylamine hydrochloride, the photometric density due to a given amount of pyridoxine is much less than when the reaction is carried out in pure solution. Thus, evaluation of the photometric density due to pyridoxine, by interpolation on a reference curve derived from pure solutions of the vitamin after correction for the borate blank, would yield erroneously low values. However, the photometric density per unit of pyridoxine (K value) in a given solution is a constant. The interferences noted are undoubtedly due to the removal of much of the 2,6-dichloroquinonechloroimide by the high concentrations of the resorcinol or naphthylamine. Despite this, the additional concentration of pyridoxine is insufficient to affect significantly the residual chloroimide, so that the K value for pyridoxine remains constant in each case. Consequently, the addition of the standard in the form of an increment to an aliquot of the test solution containing the inhibitory substance offers a means for automatically correcting for the interferences noted.

It is recognized that such a procedure constitutes a difference method

involving a sacrifice of precision. This, however, is not serious in view of the excellent reproducibility of the color development. The constancy of the K value has been repeatedly confirmed in tests of biological materials

TABLE II

Interference of Relatively High Concentrations of Other Coupling Compounds with Reaction of Pyridoxine and 2,6-Dichloroquinonechloroimide; Correction of Such Interference by Increment Procedure

Coupling compound		Added pyridoxine	Photometric density*		K value† of pyridoxine increment
			Test solution	Same + boric acid	
	γ	γ			
None	0	0	0	0	
		10	0.445	0.006	0.445
		20	0.885	0.014	0.443
Resorcinol	25	0	0.103	0.103	
		10	0.549	0.112	0.446
		20	0.983	0.117	0.440
	100	0	0.396	0.396	
		10	0.798	0.399	0.402
		20	1.199	0.411	0.402
	400	0	0.401	0.402	
		10	0.602	0.404	0.201
		20	0.817	0.407	0.208
		50	1.419	0.412	0.203
	1000	0	0.658	0.658	
		10	0.740	0.660	0.082
		20	0.816	0.661	0.079
		50	1.061	0.650	0.081
Naphthylamine hydrochloride	50	0	0.145	0.146	
		10	0 586	0.144	0.441
		20	1.026	0.149	0.440
	500	0	0.611	0.620	
		10	0.778	0.600	0.167
		20	0.954	0.614	0.172
		50	1.433	0.623	0.164
	5000	0	0 673	0.657	
		10	0.665	0.669	0.000
		20	0.680	0.660	0.004

* The colorimeter was set at 100 per cent transmission with the reagent blank. Readings were taken 60 seconds after the addition of the reagent to the solutions.

† The K value is the increase in photometric density per 10 γ of pyridoxine per tube.

(5). The advantages of the increment or internal standard procedure in assaying a wide variety of materials have also been recognized in the determination of riboflavin (8), niacin (9), and vitamin A (10).

As a result of the present studies, a procedure has been evolved for the determination of pyridoxine in test materials. This involves coupling of the vitamin with the 2,6-dichloroquinonechloroimide reagent in a single phase system, correction for the presence of other compounds yielding a blue color by the use of borate which renders the pyridoxine non-reactive, and evaluation of the resultant color by comparison with the increment in photometric density produced when a known quantity of the added vitamin is allowed to react in the same test extract.

There are other features of this procedure which make it superior to published methods based on the same coupling reaction. The rate of the reaction is dependent upon the nature of the solvent, its water content, its salinity, and the kind and concentration of the bases present (4, 11). The earlier procedures (11, 2) involve color development in a weakly buffered, generally 2-phase water-butanol system, without control of the salinity of the extract tested. The color developments are time-consuming, non-linear, and non-reproducible in replicate determinations. These procedures, therefore, require a series of standards with each determination.

The method described in this report involves a simple quantitative coupling reaction in a 1-phase system. The use of a strong ammonia-ammonium chloride buffer of high basicity and salinity eliminates completely the interferences reported (4, 11) in similar reactions, due to the different kinds and variable amounts of bases and salts in the test extracts and the degrees to which they are buffered. The reaction between the vitamin and reagent is now very rapid and more than 3 times as sensitive as before (11).

Probably the most serious objection to the procedures previously reported (11, 2), including those with other phenol reagents (1, 16), is the absence of a quantitative blank to correct for the presence of interfering phenols, amines, and other compounds which couple with chloroimide. Scudi has reported (11) unsuccessful attempts to apply a quantitative borate blank for this purpose. The value of his blank was restricted to the qualitative recognition of interfering compounds. When present in test materials, these had to be removed. In most cases (13) this was not possible, requiring the quantitative use of a qualitative blank, as a first approximation, rather than none at all. All these earlier methods depend for specificity entirely upon the preparation of extracts of biological materials free from interfering, coupling compounds. Our experiences with these procedures indicate that this is never attained with complete recovery of added pyridoxine in the assay of natural products. A compensating error may operate to some extent in these methods, since no provisions are made to correct for the possible presence of compounds which inhibit the reaction between pyridoxine and reagent.

SUMMARY

By studies conducted with pure systems the basis is established for a chemical method for the determination of pyridoxine. The vitamin couples with 2,6-dichloroquinonechloroimide in a 1-phase system. The use of a strong ammonia-ammonium chloride buffer of high basicity and salinity eliminates completely the interference due to the different kinds and amounts of bases and salts in the test solutions and the degrees to which the latter are buffered. The color reaction between the vitamin and reagent reaches maximal intensity in 60 seconds and is more than 3 times as sensitive as previously attained. By the incorporation of a borate blank, the reaction is made specific for pyridoxine. The influences of compounds which affect the rate or extent of formation of the pigment and possibly its stability have been eliminated by the increment.or internal standard procedure. This involves evaluation of the color due to the pyridoxine in a test solution by means of the increment in photometric density resulting from the reaction of an added, known quantity of the vitamin.

BIBLIOGRAPHY

1. Bina, A. F., Thomas, J. M , and Brown, E B., *J. Biol. Chem.*, **148,** 111 (1943).
2. Bird, O. D., Vandenbelt, J M., and Emmett, A. D., *J. Biol Chem.*, **142,** 317 (1942).
3. Böeseken, J , *Rec. trav chim. Pays-Bas*, **40,** 553, 568, 578 (1921).
4 Gibbs, H. D., *J. Biol. Chem.*, **72,** 649 (1927).
5. Hochberg, M., Melnick, D., and Oser, B. L , *J. Biol Chem.*, **155,** 119 (1944).
6. Hochberg, M., Melnick, D , and Oser, B. L , *J. Biol Chem.*, **155,** 129 (1944).
7. Hochberg, M., Melnick, D , Siegel, L., and Oser, B L , *J Biol. Chem.*, **148,** 253 (1943).
8. Hodson, A. Z., and Norris, L. C., *J. Biol Chem.*, **131,** 621 (1939).
9. Melnick, D , *Cereal Chem* , **19,** 553 (1942).
10. Oser, B. L., Melnick, D., and Pader, M , *Ind. and Eng. Chem.*, *Anal. Ed.*, **15,** 724 (1943).
11. Scudi, J. V., *J. Biol. Chem* , **139,** 707 (1941).
12. Scudi, J. V., Bastedo, W A , and Webb, T. J., *J. Biol Chem* , **136,** 399 (1940).
13. Scudi, J. V., Buhs, R. P., and Hood, D. B , *J. Biol. Chem.*, **142,** 323 (1942).
14. Siegel, L., Melnick, D., and Oser, B. L , *J. Biol. Chem.*, **149,** 361 (1943).
15 Stiller, E. T., Keresztesy, J. C , and Stevens, J. R., *J. Am. Chem. Soc.*, **61,** 1237 (1939)
16. Swaminathan, M., *Nature*, **145,** 780 (1940).
17. Unna, K., *Proc. Soc Exp Biol and Med.*, **43,** 122 (1940).

CHEMICAL DETERMINATION OF PYRIDOXINE IN BIOLOGICAL MATERIALS AND PHARMACEUTICAL PRODUCTS. THE MULTIPLE NATURE OF VITAMIN B₆

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Comparative assays on the same samples by biological (5, 6) and microbiological (1, 4, 10, 13) procedures for the determination of vitamin B₆ fail to show good agreement (4, 16) because of the variable vitamin B₆ activity of compounds other than pyridoxine for the test organisms employed. The use of *Streptococcus lactis* for the assay of the vitamin in natural products may give values several hundred times as great as those obtained by other methods, owing to the activity for this organism of a metabolite of pyridoxine, pseudopyridoxine (16). This compound is similar to the vitamin in its behavior towards adsorbents, eluting agents, and acids and bases, but the vitamin is almost inactive as a growth factor for *S. lactis* (14). Microbiological assays based on the growth of *Lactobacillus casei* (10) are complicated by the response of the organism to oxidative degradation products of pyridoxine (4), and hence assays of food products also give erroneously high results, many times greater than those obtained by the rat growth method (5). Alanine stimulates the growth of both *S. lactis* and *L. casei* (15). Both the yeast (1, 13, 19) and rat growth (5, 6) methods are unaffected by pseudopyridoxine (4). Values obtained by yeast growth are generally lower than those yielded by rat assays, though the results are of the same order of magnitude (4, 16).

The published chemical assay methods (2, 3, 7, 11, 17, 18) claim to measure only pyridoxine. However, the validity of the results obtained by these procedures has depended upon the quantitative separation of pyridoxine from the great variety of phenols, amines, and other reacting compounds present in biological materials which couple with the reagent. Experience with these procedures indicates that complete separation is never attained.

In the preceding paper (7), a rapid, precise, linear, and specific color reaction was described which eliminates the necessity of preparing an extract free from interfering coupling compounds. The present report describes the application of the method to the determination of pyridoxine in biological materials and pharmaceutical products. Evidence is also presented showing that compounds other than pyridoxine are found in natural materials possessing the biological activity of vitamin B₆.

Principle of Method for Biological Materials

Bound pyridoxine (12,13) is hydrolyzed by heating with strong hydrochloric acid. The free vitamin is adsorbed from solution at pH 3, and eluted with sodium hydroxide. The eluate is clarified by precipitation with isopropanol. Three aliquots of the supernatant are then tested with 2,6-dichloroquinonechloroimide reagent, one alone, another in the presence of added pyridoxine (internal standard), and the third in the presence of boric acid which renders the vitamin non-reactive without affecting the ability of other reacting compounds to couple with the reagent.

Reagents—

Chloroimide reagent (7).

Ammonia-ammonium chloride solution (7).

Boric acid solution (7).

Pyridoxine hydrochloride solution (7).

Hydrolytic agent, 4 N hydrochloric acid solution.

Buffer solution at pH 3. 73 gm. of disodium phosphate dihydrate plus 167 gm. of citric acid are dissolved in water and diluted to 1000 cc.

Adsorbent, Lloyd's reagent (obtained from Eli Lilly and Company, Indianapolis, Indiana).

Acidulated water, 0.001 N hydrochloric acid.

Eluting solution, 2.0 N sodium hydroxide.

Solutions for pH adjustments, 18 N and 1 N sodium hydroxide and 12 N and 1 N hydrochloric acid solutions.

Procedure

Throughout the determination, unnecessary exposure of the test solution to light must be avoided (8,9). However, quantitative results may be obtained if manipulations are conducted uninterruptedly in the diffuse light of the laboratory.

Preparation of Test Extract—The sample, containing from 30 to 200 γ of pyridoxine and preferably 100 γ, is weighed into a test-tube calibrated at the 20 cc. mark. 10 cc. of 4 N hydrochloric acid are added. A glass stirring rod with a loop at one end is inserted to facilitate mixing and pH adjustments in the narrow tube. The latter is immersed in a boiling water bath for 1 hour with occasional stirring. This procedure constitutes an effective means for both the hydrolysis of bound pyridoxine in biological materials and for the extraction of the vitamin. The solution is cooled and neutralized to pH 3 with an outside indicator, the final adjustments being made with the 1 N sodium hydroxide and hydrochloric acid solutions. 3 cc. of the buffer solution at pH 3 are added followed by 2.5 gm. of Lloyd's reagent. The tube is stoppered and shaken occasionally for a period of 5 minutes.

The suspension is centrifuged and the supernatant discarded. The residue is dislodged, and washed once with 15 cc. of acidulated water. The finely divided adsorbent and residuum are centrifuged and the washing discarded. 5 cc. of 2.0 N sodium hydroxide solution are added, the volume made up to the 20 cc. mark with water, and the adsorbate dispersed and kept in suspension by frequent inversions for a period of about 3 minutes. The sample is centrifuged. A 10 cc. aliquot of the eluate is mixed with 50 cc. of isopropanol in a centrifuge tube and the solution centrifuged. The clear supernatant is decanted and the pH adjusted with an outside indicator to 5 to 7 with a minimal volume (few drops) of 12 N hydrochloric acid.

Color Development—The following tubes are set up, the solutions being added to each in the order mentioned to prevent the precipitation of salts: Tube 1, 6 cc. of test extract plus 2 cc. of ammonia-ammonium chloride solution plus 1 cc. of boric acid solution; Tube 2, 6 cc. of test extract plus 2 cc. of ammonia-ammonium chloride solution plus 1 cc. of water; Tube 3, 6 cc. of test extract plus 2 cc. of ammonia-ammonium chloride solution plus 1 cc. of working pyridoxine standard (10 γ). For each series, the photoelectric colorimeter[1] with a 620 mμ filter is first set at 100 per cent transmission with Tube 1, the blank, 60 seconds after the addition of 1 cc. of the chloroimide reagent. To each of the remaining tubes, the same quantity of the chloroimide reagent is added and the blue color measured exactly 60 seconds later (7). The use of Tube 1 as a blank eliminates simultaneously the necessity for correcting for absorption due to the color of the test extract, that of the reagents, and that due to the coupling of compounds other than pyridoxine which yield colored derivatives (7).

Calculations—

$$\frac{L_2}{L_3 - L_2} \times \frac{10}{6 \text{ cc.}} \times \frac{60 \text{ cc.}}{10 \text{ cc.}} \times \frac{18.5 \text{ cc.}}{W \text{ gm.}} = \text{micrograms pyridoxine per gm.}$$

In the equation, L_2 represents the photometric density ($2 - \log G$, where G is the per cent transmission) due to the pyridoxine in Tube 2 containing 6 cc. of final test extract. $L_3 - L_2$ is the increment in photometric density due to the added 10 γ of pyridoxine. W represents the weight of test sample expressed in gm., and 60/10 × 18.5 is the dilution factor. Correction has been made in the calculation for the volume of 1.5 cc. occupied by 2.5 gm. of Lloyd's reagent in a total volume of 20 cc.

In the analysis of pharmaceutical products, the optimal size of sample is one containing approximately 100 γ of pyridoxine. In the case of natural materials, aliquots containing up to 3 gm. of total solids may be treated in the manner described above. For a substance of low potency, a larger ali-

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[1] A direct reading photoelectric colorimeter is preferred to a null point type of instrument.

quot may be taken provided a proportionately larger volume of the 4 N hydrochloric acid is used for the hydrolysis. The adsorbate is subsequently transferred, with the aid of the dilute hydrochloric acid wash, to the tube, graduated at 20 cc., and the procedure continued as outlined. A further increase in the sensitivity of the method may be effected by concentrat-

TABLE I

Reliability of Chemical Procedure for Determination of Pyridoxine; Recovery Experiments and Comparisons with Other Assay Procedures

Sample	Pyridoxine found	Recovery of added pyridoxine*			Values by other procedures	
		Pyridoxine added	Total found	Recovery	Microbiological (13)	Biological† (6)
	γ per gm	γ per gm.	γ per gm	per cent	γ per gm	γ per gm.
Rice bran concentrate ...	97	60	158	102	100	100
Liver extract powder	14	40	53	98	25	30
Yeast " "	8	50	56	97	90	85
Wheat bran...	15	20	34	95	16	
Blackstrap molasses.. ...	20				25	
	γ per sample	γ per sample	γ per sample			
24 hr. basal urine (H. H.) .	85	100	172	87‡		
24 " " " (E. M.) .	45					
24 " urine after 5 mg oral dose (H. H.)	157					
24 " " " 5 " " " (E. M.)	98					
	γ per unit	γ per unit				
Multivitamin and mineral tablet	86	85		101	80	
Vitamin B complex gelatin capsule.....	490	500		98		
Multivitamin gelatin capsule...	88	85		104		
Vitamin B complex syrup, per 5 cc..	356	350		102	344	

* The amounts of pyridoxine added for the recovery tests varied from 50 to 150 per cent of the expected values based upon biological (6) and microbiological (13) analyses of the same samples.

† According to the assay method of Dimick and Schreffler (6) in which both rat growth and the cure of rat dermatitis are taken into account.

‡ The low recovery value is attributed to the low precision of the chemical assay as applied to urine, owing to the high concentrations of other coupling compounds in the final test extracts.

ing the alkaline eluate on a hot-plate to a small volume and adding a proportionately smaller volume of the isopropanol.

Typical assays for pyridoxine conducted on some biological materials and pharmaceutical preparations are presented in Table I. Although quantitative recoveries are routinely obtained, there may be found large dis-

crepancies between the chemical data (*viz.* values for yeast and liver extract powders) and those derived from microbiological and biological assay. These differences have been noted repeatedly on replicate assays on the same and similar materials.   The significance of these differences will be discussed more fully in a later section of this report.

TABLE II

Importance of Increment Procedure and Borate Blank in Determination of Pyridoxine

Test extract per tube	Photometric density*				Inactivation of increment by borate
	Borate blank*	Test	Test† minus blank	Increment per 10 γ of pyridoxine	
					per cent
Pure solution, 10 γ pyridoxine.	0.002	0.447	0.445	0.445	100
Test solution from wheat bran.....	0.448	0.601	0.153		
Same + 5 γ pyridoxine	0.448	0.763	0.315	0.324	100
" +10 " "	0 452	0.934	0 482	0 333	99
" +15 " " .	0.444	1 091	0.647	0.327	100
" +20 " " ..	0 457	1.245	0.788	0.322	99
Test solution from rice bran	0 343	0.563	0.220		
Same + 10 γ pyridoxine	0 350	1.003	0 653	0 440	98
Test solution from liver powder .	0 335	0.439	0.104		
Same +10 γ pyridoxine .	0.345	0.779	0.434	0.340	97
Test solution from yeast powder	0.292	0.402	0 110		
Same +10 γ pyridoxine	0.290	0.829	0.539	0.427	100
Test solution from basal urine (H H)	0.393	0.508	0.115		
Same +10 γ pyridoxine	0 402	0 891	0 489	0 373	98
Test solution from multivitamin capsule	0.040	0.420	0 380		
Same +10 γ pyridoxine .	0.042	0 860	0.818	0.440	100

* In order to evaluate the borate blank the colorimeter was set for these readings at 100 per cent transmission with a tube containing 5 cc. of isopropanol, 2 cc of water, and 2 cc. of ammonia-ammonium chloride solution 60 seconds after the addition of 1 cc of chloroimide reagent

† In all cases, the blank tubes contained 1 cc. of boric acid solution.   The pyridoxine was added as 0 1 cc of stock solutions of the vitamin, so that volume changes are negligible.

The deductions drawn in the previous paper from studies with pure systems (7) are further supported by related experiments conducted with the extracts derived from these materials.   The tests deal with the importance of the increment (internal standard) procedure and the borate blank.   The findings are presented in Table II.

It will be noticed that in the analysis of natural products high blanks are observed owing to the presence of other coupling compounds.   For this reason, in the analysis of products low in pyridoxine, precise assays are

difficult to obtain. The excellent recoveries found in analyses of the liver and yeast extract powders (see Table I) may be attributed in part to the relatively large amounts of pyridoxine added for the recovery tests. These amounts were not excessive, since they varied from 50 to 150 per cent of the expected figures according to microbiological (13) and biological (6) assays on the same samples. Only in the analysis of pharmaceutical products, which contain high concentrations of pyridoxine in the presence of moderate amounts of interfering substances, are precise assays possible by the method described.

The necessity of and justification for using the increment procedure, rather than reliance on simple reference to the reaction of a standard in pure solution for the estimation of the pyridoxine content in the test extract, is demonstrated by the illustrative data for the wheat bran extract. Though the increment in photometric density per unit of pyridoxine is low compared with that for pure solutions, it is constant with varying concentrations of the vitamin.

Biologically Active Vitamin B₆ Compounds Other Than Pyridoxine in Natural Products

Scudi (12) has reported the presence of bound pyridoxine in a rice bran concentrate. Siegel, Melnick, and Oser (13) found that the vitamin occurs in nature principally in the bound form, and reported the concentrations of free and total vitamin B₆ in a variety of biological materials as determined by their modification of the microbiological method.

In the present report, three natural sources of the vitamin B complex were subjected to various extraction and hydrolytic procedures in the determination of total pyridoxine content. The results are presented in Table III.

For the chemical hydrolytic procedures, the samples were suspended in hydrochloric acid solutions of various normalities or in sodium hydroxide solution, and the test-tubes immersed in a boiling water bath for 1 hour. Total pyridoxine was then determined. 4 N hydrochloric acid at 100° for 1 hour appears to be as effective a hydrolytic agent as the previously reported procedure (13) of autoclaving in 2 N sulfuric acid at 15 pounds pressure for a period of 30 minutes. Whereas 90 per cent of the pyridoxine was in the bound form in the rice bran concentrate according to the chemical method, all of the vitamin in the dried liver powder and the dried yeast appeared to be present in the free form. Only in the assay of the rice bran concentrate were the results by the chemical method in agreement with those obtained by the biological and microbiological procedures. In every case, chemical assays of pure solutions of pyridoxine subjected to the hydrolytic procedures listed in Table III gave complete recoveries.

It is conceivable that one or more of three factors might have been responsible for the low results obtained chemically: (a) mechanical losses of the liberated pyridoxine in the course of preparing the test extract for color reaction, (b) incomplete extraction and hydrolysis of the bound pyridoxine, and (c) greater specificity of the chemical method for pyridoxine to the exclusion of other "biologically active forms" of the vitamin.

TABLE III

Liberation of Pyridoxine and Other Biologically Active Vitamin B₆ Compounds in Assay of Natural Concentrates

Assay procedure	Extraction and hydrolysis	Pyridoxine		
		Rice bran concentrate	Liver extract powder	Yeast extract powder
		γ per gm	γ per gm	γ per gm.
Chemical*	Water; 1 hr. at 100°	9	14	9
	1 N HCl 1 hr. at 100°	57	13	9
	2 " " 1 " " "	93	13	8
	3 " " 1 " " "	92	14	9
	4 " " 1 " " "	97	14	8
	5 " " 1 " " "	98	13	9
	4 " NaOH; 1 hr. at 100°	22	14	6
	Clarase digestion (1); 48 hrs. at 45°	47	13	11
	Taka-diastase + papain digestion (2); 2 hrs. at 40°			10
Microbio-	Water; 0.5 hr. at 15 lbs. pressure (121°)	10	11	34
logical†	2 N H₂SO₄; 0.5 hr at 15 lbs pressure (121°)	100	25	90
(13)				
Biological‡	None	100	30	85
(6)				

* 1 gm quantities of the rice bran concentrate and 2 and 3 gm. samples of the liver and yeast extract powders, respectively, were suspended in 10 cc. of the aqueous, acid, and alkaline solutions for the hydrolysis In the case of the enzymic digestions, the procedures followed were those indicated by the reference citations.

† According to the yeast growth method (13).

‡ According to the bioassay method of Dimick and Schreffler (6) in which both growth response and cure of the rat dermatitis are taken into consideration.

That mechanical losses of pyridoxine do not occur in the preparation of the test extracts is shown by the excellent recovery values obtained when the vitamin is added to the original test materials prior to hydrolysis (see Table I).

The lack of a gradient in the values obtained for the liver and yeast extract powders with increasing strength of the acid hydrolytic agents is presumptive evidence that no compound similar to that found in the rice bran

concentrate was available in these products for hydrolysis to free pyridoxine. In order to check this possibility further, the samples were subjected to assay with alkaline hydrolysis of comparable normality, and enzymic digestion according to procedures which have been reported to be effective for the liberation of bound pyridoxine (1,2). No increase in the values for pyridoxine was obtained. In fact, the value for the rice bran concentrate was significantly less than that yielded by strong acid hydrolysis. This low figure after clarase digestion was confirmed by replicate chemical and by microbiological assays. Thus the conclusion appears to be justified that in the liver and yeast extract powders[2] microbiologically stimulatory substances are present, and that these have an activity comparable to that of pyridoxine in promoting rat growth and in curing the dermatitis. *However, these are not pyridoxine.* That other compounds are present in the test extracts capable of reacting with the reagent is evident from the high borate blanks obtained by the chemical method. Whether or not some of these possess biological activity remains to be answered.

Abridged Chemical Procedure for Use with Pharmaceutical Products

Ascorbic acid, if present in the final test extract, interferes with the reaction of pyridoxine with 2,6-dichloroquinonechloroimide. Though the two vitamins may be separated by the use of adsorbents, this procedure is too cumbersome for use with the relatively pure systems found in pharmaceutical products containing crystalline pyridoxine. For the analysis of such preparations it has been found expedient to oxidize the ascorbic acid present by shaking the alkaline extract with manganese dioxide. The efficacy of this step is illustrated by the results of experiments conducted with pure solutions of pyridoxine and ascorbic acid, which are presented in Table IV.

In the following short modification of the chemical method, hydrolytic procedures are dispensed with, and ascorbic acid is destroyed by treatment with manganese dioxide in alkaline solution. The procedure is intended for use with pharmaceutical preparations and is precise to ± 2 per cent. A representative sample containing between 80 and 200 γ of pyridoxine is suspended in 15 cc. of 0.5 N sodium hydroxide in a tube calibrated at 20 cc. The suspension is immersed in a boiling water bath for 15 minutes with occasional agitation. The sample is cooled, 200 mg. of manganese dioxide are added, and the volume adjusted to 20 cc. with water. The tube is shaken for 5 minutes. A 5 cc. aliquot of the suspension is added to 25 cc. of isopropanol, and the mixture is centrifuged. 6 cc. aliquots of the superna-

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[2] In testing a number of different types of yeast products in our laboratories by the three assay procedures, we found that the values for pyridoxine by the chemical method are consistently only a small fraction of the microbiological values, while the latter tend to approach but never exceed the figures estimated from the rat assays.

TABLE IV

Effect of Ascorbic Acid upon Color Reaction of Pyridoxine with 2,6-Dichloroquinone-chloroimide before and after Treatment with Manganese Dioxide

Final test solution			Photometric density
Previous treatment	Pyridoxine	Ascorbic acid	
	γ *per tube*	*mg. per tube*	
None	0	0	0
	10	0	0.445
"	10	0.25	0.442
	10	0.50	0.444
	10	1.00	0.446
	10	2.00	0.000
	10	3.00	0.000
Manganese dioxide*	0	0	0.000
	10	0	0.445
	10	5.00	0.447
	10	10.00	0.443

* Before dilution with isopropanol, the aqueous solutions of the vitamins were adjusted to 0.33 N with respect to sodium hydroxide and shaken 5 minutes with 200 mg. of added manganese dioxide

TABLE V

Determination of Pyridoxine in Pharmaceutical Preparations by Abridged Chemical Procedure

Preparation	Claimed ascorbic acid content	Pyridoxine	
		Found	Minimum claimed
	mg	γ	γ
Vitamin B complex tablet, per tablet	0	530	500
" " " " " "	0	248	250
" " " capsule " capsule.	0	256	250
" " " " " "	0	510	500
Multivitamin tablet, per tablet	200	55	50
" " " "	10	362	333
" capsule, " capsule	200	57	50
" " " "	30	206	200
Vitamin mineral tablet, per tablet	10	78	83
" " capsule, " capsule	30	303	250

tant are then tested alone, in the presence of 10 γ of added pyridoxine, and in the presence of borate, as described in a preceding section of this paper.

The reliability of this procedure is indicated by typical analyses of the pharmaceutical products shown in Table V.

SUMMARY

A specific chemical procedure is described for the determination of pyridoxine in biological products and pharmaceutical preparations. Various chemical and enzymic hydrolytic procedures for the liberation and extraction of the vitamin from natural products were examined. Chemical, microbiological, and biological data are presented indicating that pyridoxine is not the only compound in nature possessing vitamin B_6 activity for the rat and for microorganisms. An abbreviated, reliable procedure is given for the chemical determination of pyridoxine in pharmaceutical products.

BIBLIOGRAPHY

1. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.,* **15**, 141 (1943).
2. Bina, A. F., Thomas, J. M., and Brown, E. B., *J. Biol. Chem.,* **148**, 111 (1943).
3. Bird, O. D., Vandenbelt, J. M., and Emmett, A. D., *J. Biol. Chem.,* **142**, 317 (1942).
4. Carpenter, L. E., and Strong, F. M., *Arch. Biochem.,* **3**, 375 (1944).
5. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.,* **138**, 555 (1941).
6. Dimick, M. K., and Schreffler, C. B., *J. Nutr.,* **17**, 23 (1939).
7. Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.,* **155**, 109 (1944).
8. Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.,* **155**, 129 (1944).
9. Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.,* **148**, 253 (1943).
10. Landy M., and Dicken, D. M., *J. Lab. and Clin. Med.,* **27**, 1086 (1942).
11. Scudi, J. V., *J. Biol. Chem.,* **139**, 707 (1941).
12. Scudi, J. V., *J. Biol. Chem* , **145**, 637 (1942).
13. Siegel, L., Melnick, D., and Oser, B. L., *J. Biol. Chem.,* **149**, 361 (1943).
14. Snell, E. E., *Proc. Soc. Exp. Biol and Med.,* **51**, 356 (1942).
15. Snell, E. E., *Proc. Soc Exp. Biol. and Med* , **55**, 36 (1944).
16. Snell, E. E., Guirard, B M., and Williams, R. J., *J. Biol. Chem.,* **143**, 519 (1942).
17. Swaminathan, M., *Nature,* **145**, 780 (1940).
18. Swaminathan, M., *Indian J. Med. Res.,* **29**, 561 (1941).
19. Williams, R. J. Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub., No. 4137,* 24 (1941).

ON THE STABILITY OF PYRIDOXINE

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Much less has been reported on the stability of pyridoxine[1] than on the other vitamins that have been isolated in crystalline form. Such information is essential in studies of pyridoxine metabolism, in assays for the vitamin, and in the preparation and preservation of pharmaceutical products.

In the present paper are presented data on the effect of light, heat, acids, alkali, and oxidizing agents on pyridoxine. The stability of the vitamin in mixed vitamin preparations subjected to storage at an elevated temperature has also been investigated.

EXPERIMENTAL

Destruction of Pyridoxine by Light

In the course of our investigations on the determination of pyridoxine, a marked instability of the vitamin to light was noted (7). This phenomenon, observed possibly by others (4, 8) in biological assays,[2] has been investigated quantitatively in the present study by the chemical method (5, 6), and by physical and microbiological methods (9) of assay.

The chemical (5, 6) and microbiological (9) methods are those described in previous papers from this laboratory. The physical method was dependent upon comparative ultraviolet absorption curves for the test solutions. A preliminary report of these findings has appeared (7).

In these experiments, absorption curves were obtained[3] at pH 6.80 for freshly prepared and irradiated solutions. Extinction coefficients at the absorption maximum, 324 mμ, were used to estimate the concentrations of pyridoxine, and the extinction ratios (assuming $E_{1cm.}^{1\%}$ 324 mμ = 1.00) employed to define the shapes of the absorption curves. The plotted curves (Figs. 1 and 2) extend from 295 to 345 mμ, and each represents measurements taken at fifteen or more wave-lengths. In all cases the irradiated

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[1] In the present report, the terms pyridoxine and pyridoxine hydrochloride are used interchangeably, and all values are expressed as the hydrochloride.

[2] The loss of vitamin B$_6$ activity for the rat on irradiation of natural concentrates has been reported by others (4, 8). Whether or not this was due to destruction of pyridoxine *per se* is questionable, particularly in view of the statement made (4) that "the fall in vitamin B$_6$ activity also occurs in the specimens irradiated at strongly acid or alkaline reactions." Pyridoxine in acid solution is stable to light (see the text).

[3] A Beckman spectrophotometer (2) was used for these measurements.

solutions initially contained 25 γ of pyridoxine hydrochloride per cc.   In
the experiments with artificial light, the solutions were exposed in an open
beaker 8 inches below a 300 watt tungsten lamp mounted in a white reflec-
tor.   Water lost by evaporation was replaced every 2 hours.   The tempera-
ture of the solutions rose to only 35–40°.   Control solutions of varying pH
kept in the dark even at 90° for 24 hours showed no pyridoxine losses.   In
the experiments with natural light, the solutions were placed in stoppered
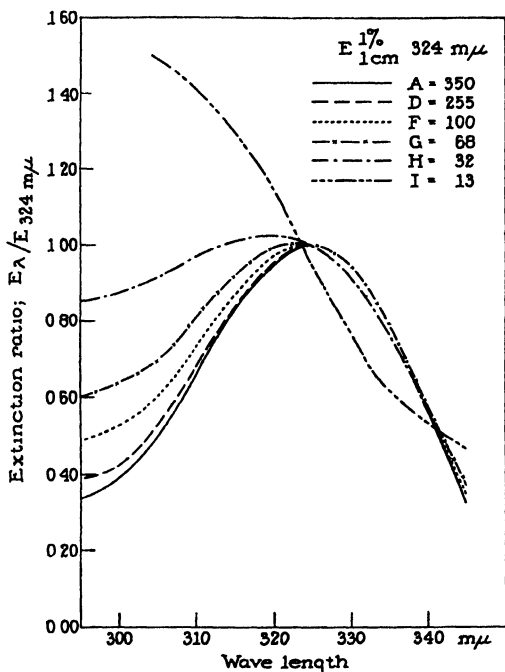
Fig. 1. Extinction ratios of aqueous solutions of pyridoxine at pH 6.8 initially
containing 25 γ per cc. and irradiated with a 300 watt tungsten lamp for varying
periods of time.   Curve A represents the freshly prepared solution; Curves D, F, G,
H, and I, the solutions irradiated 4, 14, 20, 28, and 52 hours respectively.

Erlenmeyer flasks and subjected to bright, diffuse daylight.   The tempera-
ture range of these solutions was 15–20°.

The results of the irradiation experiments are presented in Figs. 1 and 2
and Table I.   Rapid destruction of the vitamin by light in neutral and
alkaline solutions is apparent from the values obtained by all three methods
of measurement.   On the other hand, little loss is observed in 0.1 N hydro-
chloric acid (pH 1.0).   Similar results were obtained when the irradiations
were performed in an atmosphere of nitrogen.   This indicates that the
destruction was not due to photolytic oxidation.

The agreement between the chemical and microbiological methods of assay employed for these tests is excellent. Apparently both methods are specific for the determination of pyridoxine in the presence of its photolytic degradation products. Our observations (7) on the destruction of the vitamin by alkaline irradiation have recently been confirmed by Carpenter and Strong (1). They find, however, that the degradation products stimulate growth of *Lactobacillus casei*. In view of this observation, the chemical data for Experiment N in Table I are of interest. This sample contained
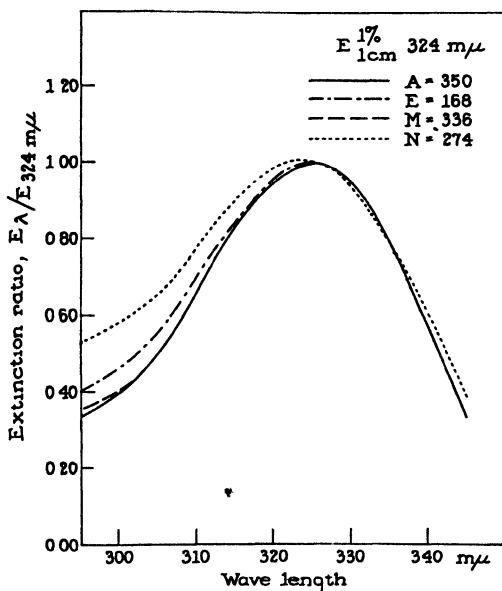
FIG. 2 Extinction ratios of aqueous solutions, readjusted to pH 6.8, of pyridoxine initially containing 25 γ per cc and irradiated with a 300 watt tungsten lamp for a period of 9 hours at varying pH values. Curve A represents the non-irradiated control solution at pH 6 8; Curves E, M, and N, the solutions irradiated at pH 6 8, 1.0, and 13.0 respectively.

high concentrations of compounds which coupled with chloroimide in the presence of borate. Thus, omission of the borate blank in this case would have given a value for pyridoxine 60 per cent higher than the true value, a figure in good agreement with that obtained from the extinction coefficient.

It may be seen in Fig. 2 that the presence of such compounds produces only moderate distortion of the pyridoxine absorption curve in the region of the ultraviolet measured.

Decomposition products coupling with chloroimide were not present in the pyridoxine solutions irradiated at an acid or neutral pH. Only when

the absorption curves were considerably distorted was poor agreement observed between the values obtained spectrophotometrically and by the chemical and microbiological methods. It is of interest that the distortions

TABLE I

Destruction of Pyridoxine by Light in Aqueous Solution

Experiment	Irradiation			Residual pyridoxine		
	Hrs.	pH	Type*	Calculated from extinction coefficient†	By chemical test	By micro-biological test
				γ per cc	γ per cc.	γ per cc
A	0	6.8	Artificial	25	25	25
B	1	6.8	"	23	23	
C	2	6.8	"	21	21	
D	4	6.8	"	18	18	
E	9	6.8	"	12	12	14
F	14	6.8	"	7	7	
G	20	6.8	"	5‡	4	
H	28	6.8	"	2.4‡	1.7	
I	52	6.8	"	0.8‡	0.3	0.1
A	0	6.8	Natural	25	25	25
J	12	6.8	"	17	17	
K	24	6.8	"	13	13	13
L	36	6.8	"	11	11	
E	9	6.8	Artificial	12	12	14
M	9	1.0	"	24	24	22
N	9	13.0	"	20‡	11§	12

* In the irradiation experiments with artificial light, the solutions were exposed in an open beaker 8 inches below a 300 watt tungsten lamp mounted in a white reflector. In the experiments with natural light, the solutions were exposed to bright diffuse daylight.

† The extinction coefficients were calculated from the optical densities at 324 mμ.

‡ Distorted absorption curves were obtained for these solutions owing to the presence of decomposition products which also absorbed in the ultraviolet region. The values for pyridoxine calculated from the extinction coefficients are therefore erroneously high.

§ This solution showed considerable color development with 2,6-dichloroquinone-chloroimide even in the presence of borate, owing to decomposition products of pyridoxine. Correction for these was made by use of the quantitative borate blank (5).

are predominantly below 324 mμ; above this wave-length, the curves are practically superposable.

These findings on the instability of pyridoxine to light have important practical applications in assay, nutritional, and chemical studies with this vitamin. However, the destruction is not so rapid as to necessitate the use

of a dark room for the routine determination of pyridoxine by a rapid chemical procedure (5, 6). If manipulations are conducted in the diffuse light of the laboratory, and the neutral or alkaline solutions are exposed for no longer than an hour, quantitative recoveries are obtained.

Stability of Pyridoxine to Heat and Chemical Agents

Data on the stability of pyridoxine to boiling and autoclaving with strong acids and alkali are presented in Table II. Heating the vitamin for 1 hour at 100° with 5 N hydrochloric acid, sulfuric acid, or sodium hydroxide does

TABLE II

Effect of Heating Pyridoxine with Acids and Alkali

Reagent	Heat treatment		Recovery of pyridoxine
	Time	Temperature	
	hrs	°C.	per cent
Water...	1	100	100
Hydrochloric acid, 5 N	1	100	100
Sulfuric acid, 5 N .	1	100	100
Nitric acid, 1 N.. .	1	100	90
" " 2 "..	1	100	78
" " 3 " ..	1	100	78
" " 4 ". .. .	1	100	58
" " 5 ".	1	100	43
Sodium hydroxide, 5 N...... . ..	1	100	100
Water... . .	0.25	121*	100
Hydrochloric acid, 0.1 N . ..	0.25	121	100
" " 1 N	0.25	121	100
Sulfuric acid, 2 N†. ..	0.5	121	100
" " 4 "... ..	0.5	121	100
Sodium hydroxide, 0.1 N... ...	0.25	121	98
" " 1 N......	0.25	121	100

* This temperature was obtained by autoclaving at 15 pounds pressure.

† Autoclaving for 0.5 hour with 2 N sulfuric acid is a procedure recommended for the liberation of bound pyridoxine for microbiological assay (9).

not affect pyridoxine. Similar treatment with nitric acid, however, results in oxidative destruction. The decomposition products do not react with the chloroimide reagent.

Carpenter and Strong (1) have reported that autoclaving pyridoxine in 0.1 or 1.0 N sodium hydroxide for 15 minutes at 15 pounds pressure results in the formation of a substance which has 2.5 times as much growth-promoting activity for *Lactobacillus casei* as pyridoxine. By the chemical test of demonstrated specificity (5, 6), such treatment is found to be without effect on the vitamin. Pyridoxine may be autoclaved for 15 minutes at

15 pounds pressure with 1 N hydrochloric acid or sodium hydroxide, or for 0.5 hour in 4 N sulfuric acid without deleterious effect.

In Table III are presented the results of miscellaneous experiments on the stability of pyridoxine. Manganese dioxide, which is employed for the removal of ascorbic acid in the analytical determination of pyridoxine in pharmaceutical products (6), is inert towards the vitamin. Potassium permanganate destroys pyridoxine; this has been noted by others (1) using the *Lactobacillus casei* procedure. Hydrogen peroxide has been reported (1) to convert the vitamin in part to a product with enhanced activity for *Lactobacillus casei*, but with no activity for yeast. By the chemical procedure, it

TABLE III

Miscellaneous Experiments on Stability of Pyridoxine

Agent	Treatment	Recovery of added pyridoxine
		per cent
Manganese dioxide* .	1 hr., 25°	100
Potassium permanganate†	1 " 25° .	1
Hydrogen peroxide‡	4 hrs , 25°	44
Cystine§	Autoclaved 1 hr , 15 lbs , pH 7.2	78

* 200 γ of pyridoxine in 20 cc of 0 33 N sodium hydroxide were shaken with 200 mg. of manganese dioxide

† 1000 γ of pyridoxine in 50 cc of water plus 0 1 cc of 4 per cent potassium permanganate were allowed to stand 1 hour The excess permanganate was then destroyed with 1 drop of 3 per cent hydrogen peroxide The latter was immediately removed with manganese dioxide.

‡ 1000 γ of pyridoxine in 30 cc. of water plus 10 cc of 3 per cent hydrogen peroxide were allowed to stand 4 hours The excess hydrogen peroxide was destroyed with manganese dioxide.

§ 250 γ of pyridoxine, 50 mg. of cystine hydrochloride, and 250 mg of sodium acetate in 250 cc. of solution (pH 7 2) were autoclaved for 1 hour at 15 pounds pressure.

is now found that the treatment with hydrogen peroxide destroys 56 per cent of the pyridoxine. The decomposition product reacts with chloroimide in the presence of borate.

Carpenter and Strong (1) have also reported enhanced activity for *Lactobacillus casei* when pyridoxine is autoclaved with cystine at pH 7.2. As measured by the more specific chemical method, this treatment is found to result in destruction of 22 per cent of the pyridoxine. The reaction products react with 2,6-dichloroquinonechloroimide in the presence of borate. A control solution autoclaved at the same pH without the cystine showed no pyridoxine loss.

The stability of pyridoxine in pharmaceutical preparations is of practical

interest. In Table IV are presented data on the stability of the vitamin in several pharmaceutical preparations subjected to an accelerated storage test. The conditions of storage were those set forth in the specifications of the United States Federal Surplus Commodities Corporation (3) for multivitamin tablets and capsules. Whereas vitamin A, thiamine, ascorbic acid, and pantothenic acid are relatively unstable members in mixed vitamin preparations, pyridoxine must now be included with riboflavin and nicotinic acid as the more stable components.

TABLE IV

Stability of Pyridoxine in Pharmaceutical Preparations Subjected to Accelerated Holding Test

Preparation	Pyridoxine content per unit		
	Minimum claimed	Found	
		Initially	After 500 hrs. at 45°
	γ	γ	γ
Multivitamin tablet.	333	392	388
Vitamin B complex tablet . ..	50	51	53
Multivitamin capsule	200	206	212
" " ...	250	303	286
Vitamin B complex capsule	250	252	261
" mineral tablet . .	83	78	78

SUMMARY

Chemical, physical, and microbiological methods were employed in a study of the influence of visible light on pyridoxine. The vitamin is unstable when irradiated in aqueous solutions at pH 6.8 or above. At pH 1.0, pyridoxine is almost unaffected. The destruction is not due to photolytic oxidation. The vitamin is stable when subjected to heating at 100° in 5 N sulfuric or hydrochloric acid, or 5 N sodium hydroxide. Even autoclaving strong acid or alkaline solutions of pyridoxine at 15 pounds pressure is without deleterious effect. The vitamin is unstable in nitric acid solutions at 100°, probably because the latter is an oxidizing agent. Thus, permanganate and hydrogen peroxide are also capable of destroying pyridoxine, even at room temperature. Manganese dioxide, on the other hand, is without effect. Pyridoxine is stable in mixed vitamin preparations subjected to accelerated storage tests, to the same degree as riboflavin and nicotinic acid.

BIBLIOGRAPHY

1. Carpenter, L. E., and Strong, F. M., *Arch. Biochem.*, **3,** 375 (1944).
2. Cary, H. H., and Beckman, A. O., *J. Optical Soc. Am.*, **31,** 682 (1941).

3. Federal Surplus Commodities Corporation, Form S. C. P. No. 1738, program GCP (1944).
4. György, P., *Biochem. J.*, **29,** 767 (1935).
5. Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **155,** 109 (1944).
6. Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **155,** 119 (1944).
7. Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.*, **148,** 253 (1943).
8. Hogan, A. G., and Richardson, L. R., *Missouri Agr. Exp. Sta.*, *Res. Bull. 178,* 3 (1932).
9. Siegel, L., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **149,** 361 (1943).

STUDIES ON THE UTILIZATION OF CRUDE RICINUS LIPASE*

V. EFFECT OF SOME SALTS ON THE ACTIVITY OF RICINUS LIPASE

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It has frequently been observed that inorganic salts influence enzyme actions. With any one enzyme addition of a salt may increase or diminish the velocity of the enzyme reaction. In studying the effect of neutral salts upon *Ricinus* lipase, Hoyer (1), Tanaka (2), and Falk and Hamlin (3) investigated the action of manganous sulfate on castor bean lipase and found that the salt increased its activity. Furthermore, Falk (4) studied the effect of various neutral salts on the action of this lipase on ethyl butyrate. He found the change in activity to be a continuous function of the concentration of salts added. Monovalent salts, the chlorides and nitrates of barium and calcium (except for very dilute solutions), magnesium chloride and nitrate, and dilute solution of sodium sulfate were found to exert a depressing effect. Dilute solution of $BaCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, and $MnSO_4$ produced increased activity. Lithium salts had a more depressing effect than the salts of sodium or potassium. In the case of the sodium and potassium halides, fluorides had the maximum inhibiting effect, iodides next, bromides next, and chlorides least. However, there appear to be no studies in the literature on the action of cations upon the activity of the lipase catalyzing the hydrolysis of oils or fats. In the present paper thirty-six salts have been investigated under suitable conditions in order to compare their action on crude *Ricinus* lipase. Twenty-two of them are different cations combined with the same anion (nitrate), and the remainder various anions with the same cation (sodium).

EXPERIMENTAL

Preparation of Lipase—The castor oil seeds, selected with special care as to size and color, were carefully removed from the hulls, crushed as fine as possible in a mortar, and mixed thoroughly. This crude *Ricinus* lipase was analyzed for acidity and oil content to determine blanks applied to subsequent analyses.

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* Four other articles have been published in Chinese (*J. Chinese Agr. Assn.*, 176–177): I. Effect of germination of castor bean (*Ricinus communis* L.) on its lipase content; II. Factors affecting the activity of *Ricinus* lipase on the hydrolysis of oils or fats; III. The activity of *Ricinus* lipase on various oils or fats; IV. Studies on *Ricinus* lipase content of various castor beans.

Determination of Activity—About 1 gm. of castor oil, 1.4 cc. of 0.112 N H_2SO_4, and 0.6 cc. of distilled water were mixed thoroughly with about 0.30 to 0.32 gm. of enzyme in a low form test-tube.   To this, 1 cc. of 0.01 N salt solution (for cations nitrate salts were used; for anions, sodium salts) was added and the mixture was incubated at 40° for 6 hours.   After

TABLE I

Effect of Cations on Activity of Crude Ricinus Lipase

Salts used, 0 01 N	Oil	Enzyme	Oil in enzyme	Total oil	Alkali for blank	Total NaOH	NaOH, net	Per cent hydrolysis	Per cent stimulation or inhibition*
	gm	gm.	gm.	gm	m eq.	m.eq	m.eq.		
AgNO₃ .	1.0240	0.3072	0.2117	1.2357	0.2623	0.2596	0		−100
Al(NO₃)₃ 9H₂O.....	1.0164	0.3049	0 2102	1.2266	0.2624	3.6098	3.3474	92.43	+21.78
Ba(NO₃)₂ .	1 0284	0.3085	0.2126	1 2410	0.2626	3.1631	2.9445	79.16	+4.29
Bi(NO₃)₃·6H₂O .	1.0350	0 3105	0.2140	1 2490	0.2631	2 3493	2 0862	56.57	−25.48
Ca(NO₃)₂	1.0300	0 3090	0.2130	1 2430	0.2627	3 1613	2.8986	78.98	+4.06
Cd(NO₃)₂ .	1 0120	0.3014	0 2056	1 2176	0 2610	3 2119	2.9409	80.23	+5.70
Co(NO₃)₂ 6H₂O ...	1.0150	0 3045	0.2099	1 2249	0 2616	3 0320	2.7704	76 60	+0.92
CuNO₃	1.0289	0.3090	0 2130	1.2419	0.2626	0 9210	0.6590	17.98	−76 73
Cr(NO₃)₃ 9H₂O	1.0292	0 3088	0.2129	1 2421	0.2627	3 5890	3.3263	90 70	+19.50
Fe(NO₃)₂ .	1.0120	0 3014	0 2056	1 2176	0 2610	3 2109	2 9499	80.43	+5.96
Fe(NO₃)₃·9H₂O .	1.0022	0 3007	0 2073	1 2095	0.2607	3 5687	3 3680	92 63	+22.04
HgNO₃·H₂O	1 0300	0.3090	0.2130	1.2430	0 2627	0 9285	0 6658	18.14	−76.10
Hg(NO₃)₂	1 0408	0 3122	0 2153	1 2651	0.2635	1 2634	0.9999	26 96	−64.63
Mg(NO₃)₂·6H₂O	1.0578	0 3173	0.2186	1.2734	0.2645	3 2427	2.9782	79.19	+4.34
Mn(NO₃)₂ 6H₂O	1.0090	0 3027	0 2087	1 2177	0 2612	3.2490	2.9878	83.10	+9.49
NaNO₃ ...	1.0288	0.3086	0.2127	1 2415	0 2627	3.1951	2.9324	80.10	+5 53
NH₄NO₃	1.0131	0 3014	0.2056	1.2187	0 2604	3 2477	2 9873	81 50	+7.38
Ni(NO₃)₂·6H₂O ..	1.0190	0.3057	0.2107	1.2297	0 2619	2.9084	2.6465	72 89	−3.01
Pb(NO₃)₂ .	1 0120	0 3036	0 2093	1.2213	0.2614	3.2407	2.9793	82 62	+8.85
Sn(NO₃)₂ .	1 0121	0.3015	0.2057	1.2178	0 2612	3.2163	2.9551	80.60	+6.21
Sr(NO₃)₂ 4H₂O	1 0370	0 3111	0 2154	1.2515	0.2632	3.3657	3.1025	83 69	+10.36
Zn(NO₃)₂ ...	1.0121	0.3015	0 2056	1.2177	0 2602	3 2953	3.0351	82 80	+9.09
Control .	1.0347	0.3104	0 2140	1 2487	0 2631	3 0316	2.7685	75.90	

* + indicates stimulation; −, inhibition.

incubation, the mixture was transferred to a 250 cc. Erlenmeyer flask with 50 cc. of neutral ethyl alcohol, and the fatty acid produced was titrated against standard alkali (0.1 N NaOH).   The results were calculated by the following formula,

$$\% \text{ hydrolysis} \quad \frac{(T - O - A - C) \times 56.1 \times 100}{W \times S}$$

TABLE II

Effect of Anions on Activity of Crude Ricinus Lipase

Salts used, 0 01 N	Oil	Enzyme	Oil in enzyme	Total oil	Alkali for blank	Total NaOH	NaOH, net	Per cent hydrolysis	Per cent stimulation or inhibition*
	gm	gm.	gm.	gm.	m eq	m eq	m.eq.		
NaF	1 0219	0 3066	0.2113	1 2332	0 2622	2.6326	2.3704	65.10	−10.86
NaCl	1.0272	0 3082	0 2124	1 2396	0.2625	3.1202	2 8577	78.08	+2.12
NaBr .	1 0278	0 3084	0.2126	1 2404	0 2626	3 0350	2 7724	75.70	−0.26
NaI.	1.0126	0.3039	0 2095	1.2221	0.2614	3.0536	2.7922	77.38	+1 42
Na₂CO₃.	1.0155	0.3048	0 2102	1.2257	0 2616	2.9604	2.6988	74.58	−1.38
Na₂SO₄ 10H₂O	1 0209	0.3063	0 2111	1 2320	0 2620	3.1245	2.8625	78.66	+2.70
Na₃PO₄	1 1084	0.3324	0 2292	1 3376	0 2684	2.6926	2.4242	61.38	−14 58
NaNO₃	1 0292	0 3087	0 2128	1 2420	0 2627	3 0136	2.7509	74 99	−0 97
Na₂CrO₄	0 9996	0.3000	0.2068	1 2064	0 2605	2·4462	2.1857	61 36	−14.60
Na₂S₂O₃·2H₂O	1 0210	0.3063	0.2111	1 2321	0 2620	3 0172	2.7552	75.74	−0.22
NaClO₃	1 0196	0 3060	0 2109	1 2305	0 2620	3 0457	2 7837	76 62	+0.66
NaCHO₂	1 0309	0 3093	0 2132	1 2441	0 2628	2 4798	2 2170	60.36	−15.60
Na₂C₂H₃O₂	1.0312	0 3093	0 2132	1 2444	0 2628	2.8491	2 5863	70 39	−5 57
Na₂C₂O₄	1.0601	0 3180	0 2192	1.2793	0 2649	3 2128	2 9479	78.05	+2.09
2Na₃C₆H₅O₇ 11H₂O	1 0148	0 3045	0 2099	1 2247	0 2616	2 7378	2 4762	68.48	−7.48
Control	1 0734	0 3220	0 2270	1 3054	0.2658	3 1935	2 9277	75 96	

* + indicates stimulation; −, inhibition.

where T = total NaOH used, in milliequivalets
  O = acidity of oil taken, in milliequivalents
  A = milliequivalents of acid added
  C = acidity of enzyme used, in milliequivalents
  W = weight both of added oil and oil in the enzyme
  S = true saponification value of the oil

Results

The effect of thirty-six salts on the activity of *Ricinus* lipase has been studied in two series. One of them is for cations and the other for anions. The results obtained are presented in Tables I and II respectively.

DISCUSSION

From the experimental data it is obvious that most cations had a stimulating effect, while the anions had an inhibiting action.

For the cations shown in Table I, a lyotropic series may be made as follows:

Fe^{+++}, Al^{+++} > Cr^{+++} > Sr^{++} > Mn^{++} > Pb^{++}, Zn^{++} > NH_4^+ > Sn^{++} > Fe^{++} > Cd^{++} > Na^+ > Ba^{++} > Mg^{++} > Ca^{++} > Co^{++} > control > Ni^{++} > Bi^{+++} > Hg^{++} > Hg^+ > Cu^+ > Ag^+

These results indicate that the cations listed before the control (no added salts) were the most effective salts in increasing the activity of lipase, while those following the control were the most effective salts in decreasing it.

In order to simplify the series, it is possible, by a careful study of these results, to point out series of regularities. For instance the effect of trivalent ions such as ferric was greater than that of divalent ions, and that of the latter was in turn greater than that of monovalent ions. Thus for stimulation the order is $C^{+++} > C^{++} > C^+$. It is just the reverse for inhibition; *viz.*, $C^+ > C^{++} > C^{+++}$. These regularities can be used either for different elements or the same element: for different elements as $Fe^{+++} > Mn^{++} > Na^+$ (in stimulation) and $Cu^+ > Hg^{++} > Bi^{+++}$ (in inhibition); for the same element as $Fe^{+++} > Fe^{++}$ and $Hg^+ > Hg^{++}$.

In industry, manganese (especially $MnSO_4$) is commonly used for stimulating the activity of *Ricinus* lipase on the hydrolysis of oils or fats. But from our experimental results, it is necessary to suggest that the ferric or similar salts must be used instead of manganese salts.

On the other hand, the anions, shown in Table II, had an inhibiting action and a series may also be given.

$$CHO_2^- > CrO_4^{--} > PO_4^{---} > F^- > C_6H_5O_7^{---} > C_2H_3O_2^- > CO_3^{--} > NO_3^- >$$
$$I^- > control > ClO_3^- > Br^- > C_2O_4^{--} > Cl^- > SO_4^{--}$$
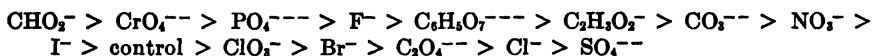
From the above series, it is clear that the general antiseptics (formate and fluoride) are powerful inhibiting agents on *Ricinus* lipase. Other ions had inhibiting effects apparently in the order of the valency of the anions; *e.g.*, PO_4^{---}, $C_6H_5O_7^{---}$, CO_3^{--}, NO_3^-, Cl^- This may also be written as $A^{---} > A^{--} > A^-$. Furthermore, from a careful study on halides, the retardation increased in the order of chloride, bromide, iodide, and fluoride.

In some cases, the anions following the control have shown a stimulating action on the lipase. This may be explained by assuming that the stimulating effect of sodium ions is greater than the inhibiting action of the anions. Consequently, the positive and negative constituents of each salt apparently exert their individual actions which sum up to give the total action of the salt.

SUMMARY

The effect of thirty-six salts on the activity of *Ricinus* lipase was studied under suitable conditions. Two lyotropic series have been determined, one for cations and another for anions.

In the case of cations, most of them increase the activity of lipase, while some diminish it. From these results a general rule according to valency has been suggested, thus $C^{+++} > C^{++} > C^+$ for stimulation and $C^+ >$

$C^{++} > C^{+++}$ for inhibition. The rule for most cases can be used either for different elements or the same element.

On the other hand, the anions mostly decrease the activity of lipase, but in a few cases such as ClO_3^-, Br^-, etc., show an increasing action. However, this effect may be due to the stimulating effect of the cation (Na) being greater than the inhibiting action of the anions. Hence a general rule for the inhibiting action of anions is $A^{+++} > A^{++} > A^+$.

BIBLIOGRAPHY

1. Hoyer, E., *Z. physiol. Chem.*, **50**, 414 (1906–07).
2. Tanaka, *Orig. Communicat. VIII Internat. Cong. Appl. Chem.*, sect. 2, 37 (1912).
3. Falk, K. G., and Hamlin, M. L., *J. Am. Chem. Soc.*, **35**, 210 (1913).
4. Falk, K. G., *J. Am. Chem. Soc.*, **35**, 601 (1913).

FATTY ACID METABOLISM. THE MECHANISM OF KETONE BODY SYNTHESIS FROM FATTY ACIDS, WITH ISOTOPIC CARBON AS TRACER*

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In a recent communication (1), results were reported of a study of the breakdown of n-octanoic acid labeled by incorporation of an excess of the heavy carbon isotope, C^{13}, in the carboxyl group. Proof was offered for a mechanism of ketone body formation involving the random coupling of identical 2-carbon units formed by β oxidation of the octanoic acid. The present paper is concerned with a more detailed description of these experiments and a discussion of the significance of the results obtained to the general question of the oxidation pathways of fatty acids.

EXPERIMENTAL

Preparation of Carboxyl-Labeled n-Octanoic Acid—Carbon dioxide was prepared by the oxidation of 0.49 gm. (0.01 mole) of isotopic sodium cyanide with aqueous potassium permanganate. The CO_2 was released by means of sulfuric acid into a high vacuum system, where it was purified and dried by repeated sublimation at $-80°$ and condensation at $-200°$. Analysis in the mass spectrometer gave a C^{13} content of 5.51 per cent.[1] The CO_2 was then condensed into a highly evacuated flask containing an excess of n-heptylmagnesium bromide in ether. After several hours were allowed for the reaction to go to completion, the reaction mixture was decomposed with dilute sulfuric acid and distilled with steam. The distillate was extracted with ether, the ether extract was shaken with dilute sodium hydroxide, and the aqueous solution of sodium octanoate acidified and reextracted with ether. The extract was then dried over anhydrous sodium sulfate and the ether evaporated. The residual colorless oil weighed 1.175 gm., representing a yield of 81 per cent. A Duclaux distillation revealed no perceptible volatile acid other than n-octanoic. Neutralization equivalent, observed, 143.0; calculated, 144.2.

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* With the technical assistance of Mary Cammaroti.

[1] The C^{13} content is expressed as atoms per cent C^{13} calculated from the equation (2),

$$\text{Atoms \% } C^{13} = \frac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100$$

The normal C^{13} complement for our instrument was found to be 1.04 per cent.

A specimen of this acid was oxidized according to the wet combustion method of Van Slyke and Folch (3). The resultant CO_2 had a C^{13} content of 1.59 per cent, corresponding to a C^{13} content for the carboxyl carbon of $(1.59 \times 8) - (1.04 \times 7) = 5.44$ per cent, in excellent agreement with the value of 5.51 per cent found for the C^{13} content of the original CO_2.

Incubation with Liver Slices—Liver slices of 0.4 mm. thickness from rats fasted 24 hours were shaken for 2 hours at 37.5° with 30 ml. of a 0.01 N sodium octanoate solution in a phosphate-buffered Ringer's solution. The composition of the medium was as follows: sodium octanoate 0.010 M, NaCl 0.051 M, PO_4 buffer, pH 7.4, 0.010 M, $CaCl_2$ 0.001 M, KCl 0.002 M. The reaction was carried out in Warburg type vessels of 125 ml. capacity in an atmosphere of oxygen, with 0.5 ml. of 2 N H_2SO_4 in the side cup and filter paper soaked with 0.5 ml. of 15 N NaOH in the central well. The uptake of oxygen was measured by an attached gas burette. At the end of the incubation period the acid, sufficient to bring the pH of the medium to 3.5, was tipped in from the side cup and shaking continued an additional 15 minutes to allow complete absorption of the liberated CO_2.

Determination of Respiratory CO_2—The alkali-soaked filter paper in the central well was quickly transferred to another Warburg vessel containing 20 ml. of CO_2-free water, the air was swept out with a stream of CO_2-free nitrogen, and, after equilibration, the CO_2 was liberated by acid tipped in from the side cup. The evolved CO_2 was measured volumetrically. The gases were then swept, by means of nitrogen, into an absorption tube containing barium hydroxide, from which the CO_2 was ultimately liberated by acid into an evacuated storage tube for determination of the C^{13} content.

Decomposition of Acetoacetic Acid and Isolation of Acetone and CO_2 Therefrom—The solution was decanted from the liver slices into a 50 ml. centrifuge tube, the slices were washed successively with small portions of dilute alkali and water to remove any adsorbed fatty acid, and the washings added to the solution. The soluble proteins and sugar were removed by the addition of 1 ml. of 25 per cent copper sulfate, followed by sufficient solid calcium hydroxide for alkalinity. After standing $\frac{1}{2}$ hour, the mixture was centrifuged, and the clear supernatant liquid refluxed with mercuric sulfate according to the procedure of Van Slyke (4). The CO_2 liberated by the decomposition of the acetoacetic acid was swept, by a stream of nitrogen, into barium hydroxide solution, from which it was liberated by acid into an evacuated storage tube for determination of the C^{13} content. The precipitated acetone-mercury complex was transferred to a weighed, sintered glass funnel, washed thoroughly, and dried at 110°. It was then oxidized by the wet combustion method of Van Slyke and Folch for determination of the C^{13} content.

The filtrate from the acetone precipitation was then treated with di-

chromate according to Van Slyke (4) for the oxidation of β-hydroxybutyric acid, and the acetone and CO_2 isolated as described above. C^{13} determinations were made on the CO_2 evolved during the oxidation, but the amount of acetone-mercury complex formed was too low for C^{13} determinations.

Recovery of Residual Fatty Acids—The final filtrate was diluted to 200 ml. and the volatile acids determined by the method of Friedemann (5). Duclaux distillations were carried out on the residual fatty acids for comparison with the original octanoic acid.

Isotope Analysis—The C^{13} determinations were carried out with a commercial mass spectrometer manufactured by the Consolidated Engineering Corporation of Pasadena, California. The C^{13} values given herein have

TABLE I

Distribution of C^{13} in Products of Incubation of 0.01 M Carboxyl-Labeled Octanoate with Liver Slices from Fasted Rats

Time, 2 hours; temperature, 37.5°.

Tissue, gm Volume of medium, ml	Experiment 1 1 25 30		Experiment 2 1 25 30		Experiment 3 2 50 60		Experiment 4 2 50 60	
	mM	per cent C¹³	mM	per cent C¹³	mM	per cent C¹³	mM	per cent C¹³
Octanoic acid utilized		1 59	0.207	1 59	0.389	1 59	0.309	1.59
O_2 consumed....	0 219		0.242		0.338		0.283	
CO_2 evolved in respiration		1.41	0 143	1 34	0.221	1 35	0.161	1.39
Acetoacetic acid								
Acetone	0.0383	1 33	0 0787	1.34	0.0579	1.30	0.0755	1.33
Carboxyl		1 80		1.89	0.0330	1 78	0 0659	1.85
Hydroxybutyric acid								
Acetone	0 0162		0.0143		0 0294		0.0376	
Carboxyl				1 22			0.1623	1.15

been corrected for the 0.04 per cent of O^{17} naturally present in oxygen contributing to the mass 45 peak as $CO^{16}O^{17}$.

Results

The results of four experiments in which carboxyl-labeled *n*-octanoic acid was incubated for 2 hours with liver slices from fasted rats are shown in Table I. The mean C^{13} content of the acetone moiety of the acetoacetic acid is 1.32 ± 0.02 atom per cent, representing an excess of 0.28 per cent over the normal complement of 1.04 per cent. The CO_2 derived from the carboxyl group of the acetoacetic acid contained 1.82 ± 0.03 per cent C^{13}, an excess of 0.78 per cent.

Location of C^{13} within Acetone Molecule—To ascertain the position of the C^{13} within the acetone the remaining Denigès mercury-acetone complex

from the four experiments was pooled, giving in all 40.2 mg., representing 2.01 mg. of acetone. It was dissolved in 2 ml. of 6 N HCl and the acetone distilled at room temperature under reduced pressure into a cooled mixture of 2 ml. of 15 N NaOH and 2 ml. of 0.1 N iodine solution. After warming to room temperature, the mixture was acidified. The iodoform was separated by centrifugation, washed thoroughly, and oxidized to CO_2 by the wet combustion procedure of Van Slyke and Folch. The CO_2 thus obtained had only the normal complement of C^{13}, 1.04 per cent. Since the iodoform in this procedure is derived from the methyl carbons, it is clear that the excess C^{13} of the acetone is exclusively in the carbonyl carbon. Inasmuch as the mean C^{13} content of the entire acetone molecule is 1.32, an excess of 0.28 per cent, the C^{13} excess of the carbonyl carbon is (3×0.28) or 0.84 per cent.

Respiratory CO_2—The mean value for the C^{13} content of the respiratory CO_2 was 1.37 ± 0.03 per cent, representing an excess of 0.33 per cent. Since the octanoic acid contained 1.59 per cent C^{13}, or an excess of 0.55 per cent, the average fraction of the respiratory CO_2 derived by complete oxidation of the substrate is 0.33/0.55 or 60 per cent of the total respiratory CO_2.

Disappearance of Octanoic Acid—In three of four experiments shown in Table I determinations were made of the amount of *n*-octanoic acid which disappeared during incubation. Of 0.207, 0.389, and 0.309 mM of octanoic acid utilized in these experiments only 0.0930, 0.0873, and 0.1131 mM respectively of total ketone bodies (as acetone) were recovered. Thus, of the octanoic acid disappearing, only 22, 11, and 18 per cent respectively can be accounted for as ketone bodies. Because of this surprising finding the method of recovery of the octanoic acid was carefully investigated. The residual acid was generally recovered after the successive steps of deproteinization, precipitation of acetone, and oxidation of β-hydroxybutyric acid. Preliminary experiments with known quantities of acetoacetic, β-hydroxybutyric, and *n*-octanoic acids of the same order as is dealt with in these experiments gave quantitative recoveries of each component. However, in order to rule out any possibility of loss of octanoic acid by any of these procedures, in Experiments 3 and 4 aliquots of the solution after incubation were acidified and distilled directly. The octanoic acid recovered by this procedure was not significantly greater than that obtained after deproteinization and ketone body isolation. The possibility that octanoic acid may be bound by the tissue was also investigated. A buffered 0.01 M solution of octanoic acid was divided into two equal parts, one of which was distilled directly, the other shaken with 1.25 gm. of liver slices and then distilled. The recovery of acid was quantitative in both

instances; hence there is no significant absorption of octanoic acid by the tissues.

Volatile Fatty Acids—The Duclaux constants for the initial octanoic acid and the voltaile acids obtained by direct distillation after incubation are compared in Table II. There is indicated the presence of a small amount of a more volatile acid accompanying the octanoic acid. Inasmuch as butyric and hexanoic acids can be detected by odor in very low concentrations, there is little likelihood they are present in significant amounts. That the acid is possibly acetic acid is indicated by comparison of the Duclaux constants of the volatile acids with those of a synthetic mixture consisting of 90 per cent octanoic and 10 per cent acetic acid (Table II). The constants are practically identical.

TABLE II   ·

Duclaux Constants of Fatty Acids

	Total volume distilled				
	12 5 per cent	25 0 per cent	37 5 per cent	50 0 per cent	75 0 per cent
	Per cent of total acidity in distillate				
n-Octanoic acid	88.0	96.5	97 9	98 4	100.0
Carboxyl-labeled *n*-octanoic acid	86 1	97.0	98 2	98.8	100.0
Volatile acids, Experiment 4	79 5	91.0	94.3	96 1	100.0
90% octanoic acid-10% acetic acid	79.0	91 1	93.6	95.8	100 0
Acetic acid	14 7	30.1	46 4	63 9	100.0

DISCUSSION

Mechanism of Ketone Body Synthesis—The classical theory of β oxidation (6, 7) assumes that successive β oxidation along the fatty acid chain occurs, the last 4 carbon atoms, in the form of butyric acid, being oxidized directly to the ketone bodies. As originally conceived, this theory failed to account for the absence of acetic acid, which would be expected in large amounts as a result of β oxidation (8-10), and the formation, under certain circumstances, of more than 1 molecule of ketone body per molecule of fatty acid catabolized (11). As a result, several modifications of this theory have been proposed. To account for the high yields of ketone body by liver slices Jowett and Quastel (11) revived the hypothesis of Hurtley (8) that oxidation occurs at alternate carbon atoms throughout the fatty acid chain prior to splitting into units of 4 carbon atoms. Known as the theory of multiple alternate oxidation, it has been accepted widely (10-12).

Recently MacKay (13) has suggested that the ketone bodies may be formed by condensation of acetic acid (or some other 2-carbon intermediary)

resulting from β oxidation of the fatty acid. Although no experimental evidence was offered for this hypothesis, it is consistent with the known facts on which are based the earlier theories, and it explains certain findings which cannot be reconciled with the theory of multiple alternate oxidation; namely, (a) the formation of more ketone bodies from hexanoic than from butyric acid (12), and (b) the formation of ketone bodies from valeric acid, 3 carbon atoms of which are known to form glycogen (14, 15).

The essential correctness of the principle of β oxidation was conclusively demonstrated by Schoenheimer who found, with deuterium-containing fatty acids, the mutual interconversion *in vivo* of palmitic and stearic acids (16).

The presence of excess C^{13} in the acetone portion of acetoacetic acid formed by the breakdown of carboxyl-labeled n-octanoic acid is proof that a redistribution of the carbon atoms occurred during this process, a result which is inconsistent with either the classical theory of β oxidation or the multiple alternate oxidation hypothesis. Moreover, the equal distribution

TABLE III

Comparison of Observed Values of C^{13} Distribution in Acetoacetic Acid Predicted by Various Theories of Ketone Body Formation

	Atom per cent C^{13}, excess	
	Carbonyl	Carboxyl
Observed	0.84	0.83
β oxidation-condensation theory	1 10	1.10
Multiple alternate oxidation theory	0	2.20
Classical β oxidation theory	0	0

of the excess C^{13} between the carbonyl and carboxyl carbons of acetoacetic acid and the absence of excess C^{13} from the methyl and methylene carbons are consistent only with a mechanism involving the random coupling of identical 2-carbon units. The observed distribution of the excess C^{13} between the carbonyl and carboxyl carbons, compared with the values calculated for the three theories discussed above, is shown in Table III. The correspondence between the observed values and those predicted by the β oxidation-condensation mechanism is actually better than that indicated in Table III when it is recalled that in the absence of a substrate there is a spontaneous formation of ketone bodies sufficient to account for the differences noted.

The observations also rule out the mechanism for the formation of acetoacetic acid suggested by Krebs and Johnson (17), whereby it was presumed acetic and pyruvic acids condense to form acetopyruvic acid, which is then decarboxylated, according to the equation

$$CH_3COOH + CH_3COCOOH \rightarrow CH_3COCH_2COCOOH \rightarrow CH_3COCH_2COOH + CO_2$$

The results also preclude any possibility of the formation of ketone bodies by a mechanism involving either α or ω oxidation of fatty acid (18). It should be pointed out, however, that these mechanisms, ruled out in the liver slice experiments, may occur in the intact organism.

Formation of Lower Fatty Acids—In β oxidation according to the classical theory, the assumption is made that a molecule of the next lower homologue, in this case hexanoic acid, is formed. If the latter had to compete with the remaining octanoic acid for the available active surfaces of the enzymes involved, there should occur an accumulation of hexanoic acid as oxidation proceeds. The same considerations apply to the lower acids, butyric and acetic. As a result, varying amounts of hexanoic, butyric, and acetic acids would accumulate, depending on the relative rates at which each was transformed. But these experiments gave no evidence for the presence of either hexanoic or butyric acid, and acetic acid, in agreement with previous observations (10, 14), was present, if at all, in amounts small in comparison with the amounts of octanoic acid catabolized. Several explanations may be offered for this phenomenon, though they have not as yet been put to experimental test: (a) the lower acids may be oxidized or otherwise metabolized much more rapidly than the original 8-carbon acid; (b) the fatty acids may be so adsorbed to the surface of the enzyme that complete conversion to acetic acid occurs without dissociation of the intermediate lower fatty acids.

Nature of the 2-Carbon Intermediary—Although the nature of the 2-carbon fragment formed by β oxidation is not known with certainty, acetic acid appears to be the most likely possibility, since it is the only 2-carbon compound known to form ketone bodies both *in vitro* and in intact animals (11, 13, 14). The experiments of Swendseid, Barnes, Hemingway, and Nier (19) with carboxyl-labeled acetic acid are particularly convincing.

At first glance it is difficult to reconcile this conclusion with the observation of previous investigators that acetic acid forms ketone bodies at a slower rate than the higher acids (11, 14). It is possible, however, that in these *in vitro* studies the absorption of acetic acid through the cell wall is slow and is the limiting factor in the rate of its transformation to the ketone bodies. It is possible also that the 2-carbon intermediary is not acetic acid itself, but a more active derivative, such as, for example, acetyl phosphate (20).

In view of the possible rôle of acetic acid as an intermediate in the synthesis of ketone bodies, suggested by this work, it is noteworthy that Lehninger (21) has found an enzyme in muscle mince and bacteria which catalyzes the reverse reaction, the breakdown of acetoacetic to acetic acid.

Other Possible Breakdown Pathways of Octanoic Acid—Of the octanoic acid utilized in these experiments only about one-fifth or less can be ac-

counted for by the formation of ketone bodies (Table I). The question arises, therefore, as to other possible breakdown products. One possibility is complete oxidation to CO_2. Ordinarily it is impossible to know what proportion of the respiratory CO_2 comes from the substrate and how much represents oxidation of tissue constituents. With the aid of the carbon isotope, however, the CO_2 arising from the substrate may readily be calculated. In Experiment 4, for example, the fraction of the respiratory CO_2 which would arise from complete oxidation of the octanoic acid amounts to 62 per cent (see the computation under respiratory CO_2). Of the 0.161 mM of respiratory CO_2 obtained 0.161 × 0.62 or 0.0998 came from the substrate. Since each mole of octanoic acid yields 8 moles of CO_2 upon complete oxidation, the 0.0998 mM of CO_2 represents the complete oxidation of 0.0998/8 = 0.0125 mM or only (0.0125 × 100)/0.309 = 4.0 per cent of the total octanoic acid which disappeared. Similar calculations for Experiments 2 and 3 give values of 4.7 and 4.0 per cent respectively. Inasmuch as a portion of the respiratory CO_2 came from spontaneous decarboxylation of the acetoacetic acid, the portion arising by oxidation is probably lower than the calculated values. It is clear that only a small amount of octanoic acid was completely oxidized.

This conclusion is borne out by consideration of the oxygen consumed, which in each experiment was very much less than would be required if extensive oxidation of the octanoic acid had occurred. The results suggest that octanoic acid may be catabolized by some as yet unknown non-oxidative reaction, or its oxidation may be coupled with a reductive process. No products containing C^{13}, other than the ketone bodies, have as yet been isolated from these experiments, but the investigation is continuing.

SUMMARY

1. When n-octanoic acid, labeled by the incorporation of C^{13} in the carboxyl group, was incubated in vitro with liver slices from fasted rats, the resultant acetoacetic acid contained the excess C^{13} equally distributed between the carbonyl and carboxyl carbon atoms. The results offer unequivocal evidence that the ketone bodies are formed by condensation of a 2-carbon intermediary resulting from β oxidation of the fatty acid.

2. Although the nature of the 2-carbon intermediary formed by β oxidation is unknown as yet, the most probable substance is acetic acid.

3. Only 20 to 25 per cent of the octanoic acid which disappeared could be accounted for either by ketone body formation or by complete oxidation to CO_2.

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BIBLIOGRAPHY

1. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **153**, 689 (1944).
2. Wood, H. G., Werkman, C. H., Hemingway, A , and Nier, A. O., *J. Biol. Chem.*, **139**, 365 (1941).
3. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
4. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 415 (1929).
5. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
6. Knoop, F., *Beitr. chem. Physiol. u. Path.*, **6**, 150 (1904).
7. Dakin, H. D., *J. Biol. Chem* , **6**, 221 (1909).
8. Hurtley, W. H., *Quart. J. Med.*, **9**, 301 (1915).
9. Wakeman, A. J., and Dakin, H. D , *J. Biol. Chem.*, **6**, 373 (1909).
10. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *J. Biol. Chem.*, **137**, 75 (1941).
11. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).
12. Deuel, H. J., Jr., Hallman, L F., Butts, J. S., and Murray, S., *J. Biol. Chem.*, **116**, 621 (1936).
13. MacKay, E. M., *J. Clin. Endocrinol.*, **3**, 101 (1943).
14. Leloir, L F., and Muñoz, J. M., *Biochem. J.*, **33**, 734 (1939)
15. MacKay, E. M., Wick, A N., and Barnum, C. P., *J. Biol Chem.*, **136**, 503 (1940).
16. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **120**, 155 (1937).
17. Krebs, H. A., and Johnson, W. A , *Biochem. J* , **31**, 772 (1937).
18. Witzemann, E. J., in Nord, F. F., and Werkman, C H., Advances in enzymology and related subjects, New York, **2**, 265 (1942)
19. Swendseid, M. E., Barnes, R. H , Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
20. Lipmann, F., *J. Biol. Chem.*, **134**, 463 (1940).
21. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942)

THE USE OF LACTOBACILLUS FERMENTUM 36 FOR THIAMINE ASSAY*

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For the assay of thiamine several microbiological, chemical, and physical methods have been introduced.

The yeast fermentation method of Schultz, Atkin, and Frey (1) is limited in use by the special apparatus needed, the activity of the pyrimidine[1] moiety of thiamine, and the high blank fermentation value. In the yeast growth method (2) the pyrimidine and thiazole halves of the thiamine molecule are also stimulatory. *Phycomyces blakesleeanus* (3, 4) responds to pyrimidine and thiazole when both are present, requires over 2 weeks for assays, and is the least sensitive of the microbiological agents. The use of *Streptococcus salivarius* by Niven and Smiley (5) is not practical for routine assays because the organism is grown at pH 7.4, necessitating aseptic addition of all thiamine and sample solutions to the autoclaved tubes of medium.

Colorimetric chemical methods (6, 7) are not sensitive enough for materials of low potency and present difficulties in the removal of interfering substances. The thiochrome method (8, 9) is also limited to higher concentrations of thiamine for accurate results and with extreme care gives reproducible values. In urines F_2, a fluorescent metabolite of nicotinamide, may interfere with determinations by the thiochrome method (10).

In the present communication a rapid microbiological method for thiamine assay is proposed in which *Lactobacillus fermentum* 36[2] is used. The growth response is measured turbidimetrically after 16 to 18 hours. Under the conditions of the test, pyrimidine and thiazole are not active either

alone, together, or in the presence of thiamine. The organism shows a steep quantitative response in the presence of 0.005 to 0.04 γ of thiamine per tube (10 ml.) and can be used for assay of foods, tissues, urines, and vitamin concentrates.

Method

Lactobacillus fermentum 36, which was isolated and described by Stiles, Peterson, and Fred (11), is kept in stab cultures containing 1 per cent glucose, 1 per cent Difco yeast extract, and 2 per cent agar. The stabs are incubated at 37° until heavy growth is obtained, 24 to 36 hours, and are then kept in the refrigerator. Stock cultures are transferred every month. Stabs for daily use are prepared similarly as needed. The inoculum for

TABLE I

Basal Medium

Alkali-treated peptone (+ sodium acetate)	20 gm	Salt Solution B*.	10 ml.
Acid-hydrolyzed vitamin-free casein	5 "	Riboflavin	200 γ
Glucose	40 "	Calcium pantothenate	200 "
Sodium acetate, anhydrous	12 "	p-Aminobenzoic acid	200 "
Cystine	200 mg	Nicotinic acid .	200 "
Adenine sulfate	20 "	Pyridoxine hydrochloride	200 "
Guanine hydrochloride	20 "	Biotin	0 8 "
Uracil	20 "	Folic acid†	0 5 "
Salt Solution A* .	10 ml	Distilled water to 1 liter, pH 6.5	

* Prepared according to Snell and Strong (12).

† A folic acid concentrate was kindly furnished by Dr R J Williams of the University of Texas, and is used here in terms of 40,000 potency

assay is always made from stab culture, never by subculturing. For this inoculum the organism is grown at 37° for 16 to 24 hours, but not longer, in the basal medium to which 0 1 γ of thiamine and 5 mg. of Difco yeast extract have been added.

The composition of the basal medium is shown in Table I. The addition of thiamine alone enables *Lactobacillus fermentum* to grow as rapidly and as heavily as in the presence of yeast or liver extracts.

The glucose and acetate concentrations of the medium (including the acetate in the peptone preparation) are equal to the larger amounts suggested for *Lactobacillus casei* (13) and promote faster growth. The alkali-treated peptone (14) contributes markedly to the rapid growth of this organism. The use of untreated peptone gives higher blank growth. Substitution of casein hydrolysate for the peptone permits only slow growth.

However, casein hydrolysate is stimulatory in the presence of this maximal amount of peptone and is included. The casein is prepared by autoclaving 16 hours with 25 per cent sulfuric acid, removal of the sulfate with barium hydroxide, norit treatment at pH 3 (10 gm. per 100 gm. of casein), and subsequent neutralization.

Without purines maximal growth is not obtained with larger amounts of thiamine and the curve slopes off above 0.03 γ per tube. Adenine alone restores most of this additional growth but the use of all three bases for assays seems preferable. Adenine, guanine, and uracil are kept in a solution containing 1 mg. of each per ml., prepared by heating in the presence of hydrochloric acid.

A similar situation exists with the vitamins in the basal medium. *Lactobacillus fermentum* requires only thiamine and pantothenic acid for good growth. The addition of riboflavin to these is stimulatory and, with *p*-aminobenzoic acid also included, the same 18 hour growth curve is obtained as with all the vitamins shown. However, assays of materials with a medium supplemented only with pantothenic acid, riboflavin, and *p*-aminobenzoic acid give higher thiamine values than with the complete medium and sometimes show a downward drift with larger assay samples or erratic recoveries of added thiamine These difficulties are not encountered when the complete medium is used. A concentrated solution of the seven vitamins of the medium is kept in the refrigerator and renewed each week.

Other supplements which have been tried but which were found to be unnecessary in the medium are asparagine, tryptophane, inositol, choline, and various yeast and liver extracts treated with sulfite or fullers' earth. The growth curve is not affected by the presence of 5 or 10 mg. of undigested starch per tube nor by 50 or 100 γ amounts of stearic acid, oleic acid, or Wesson oil.

Assay Procedure— Assays are carried out in 20 × 150 mm. lipless Pyrex test-tubes. The sample (at pH 6.5) in each tube is diluted (if necessary) to 5 ml. with distilled water and 5 ml. of the basal medium (pH 6.5) shown in Table I are added to each. A standard curve, which is run every time, contains 0.0, 0.005, 0.01, 0.015, 0.02, 0.03, 0.04, and 0.05 γ of thiamine hydrochloride per tube (10 ml.). Samples to be assayed are usually run at four levels, 1 to 4 ml., containing approximately 0.005 to 0.01 γ of thiamine hydrochloride per ml. All thiamine values in the present communication are in terms of thiamine hydrochloride.

The tubes are plugged and steamed at 100° for 15 minutes. This steaming is sufficient to keep the test free of contamination for the short time that it is run. Steaming, in contrast to autoclaving at higher temperature, does not darken the medium and decreases the possibility of any thiamine destruction.

After cooling, the tubes are inoculated with 1 drop of a dilute inoculum, prepared by adding 0.05 ml. of the 24 hour inoculum (previously described) to 10 ml. of sterile saline. The set is incubated at 37° for 16 to 18 hours, then cooled in a refrigerator for 15 minutes to stop growth, and the turbidity measured in any reliable photoelectric colorimeter. We have used a Pfaltz and Bauer instrument for which a 20 mm. tube holder was built. The turbidities are read with a 5400 A filter with an uninoculated tube as a blank. A typical standard curve for thiamine is shown in Fig. 1. The turbidimetric readings are given in terms of optical density which is equal to log 100 minus log per cent transmission.

The lower curve in Fig. 1 is the growth obtained with equal amounts of the pyrimidine and thiazole moieties at 18 hours.[3] The presence of either or both of these substances at these levels does not change the standard
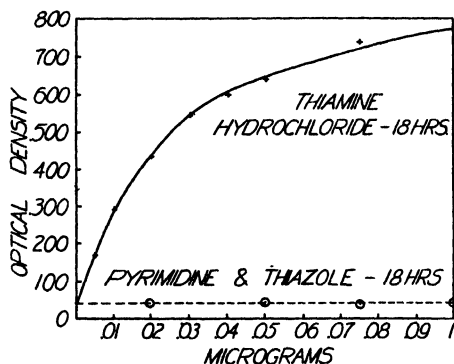
Fig 1. Growth response of *Lactobacillus fermentum* to thiamine and derivatives. Optical density as log 100 minus log per cent transmission (equals $2 - \log G$).

curve for thiamine or the response to biological extracts in 18 hours. In 20 to 24 hours *Lactobacillus fermentum* starts to use these halves of the molecule, showing from 5 to 10 per cent of the activity of thiamine, and in 40 to 48 hours is using them almost as well as it does thiamine. This prevents the use of an assay by acid titration and necessitates turbidimetric readings. These readings are best after 16 to 18 hours of incubation at 37°, although the same assay results may be obtained as early as 14 hours.

After 16 to 18 hours cocarboxylase is about 30 per cent more active than

[3] Occasionally a culture shows 5 to 10 per cent activity with pyrimidine and thiazole in 18 hours. This is checked in our laboratory by adding 0.05 γ each of pyrimidine and thiazole to a tube containing 0.01 γ of thiamine and comparing the growth obtained with that for 0.01 γ of thiamine of the standard curve. This change in response may occur if the inoculum is too concentrated or if the stab is not fully grown, in which case the stab is discarded and a new one made from stock.

equimolecular amounts of thiamine, although both compounds are equally stimulatory after 24 hours of incubation.

Preparation of Samples for Assay—For complete extraction of thiamine from foods and tissues and conversion of cocarboxylase to free thiamine it has been found necessary to digest the samples enzymatically. 1 gm. of the finely ground or blended sample is digested under benzene for 16 to 24 hours with 20 mg. each of papain and taka-diastase in 40 ml. of 0.5 per cent acetate buffer, pH 4.5 (15). The digest is then steamed, diluted to 50 ml., filtered with the aid of Celite, and the pH adjusted to 6.5 to 6.6. For

TABLE II

Thiamine Analyses and Recoveries

Material	Sample	Thiamine found	Thiamine per gm or ml	Recovery
	mg.	γ	γ	*per cent*
White flour, un-enriched	10	0.0080	0.80	
	20	0.0165	0.83	
	30	0 0243	0.81	
	40	0.0320	0.80	
	10 + 0.01 γ thiamine	0.0178		98
	20 + 0.01 " "	0.0260		95
Dehydrated pork	2 5	0.0060	24	
	5.0	0.0140	28	
	7.5	0.0210	28	
	10.0	0.0280	28	
	2 5 + 0.01 γ thiamine	0.0170		110
	5.0 + 0.01 " "	0.0230		90
	ml.			
Urine, 24 hr. sample, 1500 ml.	0.067	0.0062	0.093	
	0.133	0.0136	0.102	
	0.20	0.0204	0.102	
	0.267	0.0256	0.097	
	0 067 + 0.01 γ thiamine	0.0170		108
	0.133 + 0.01 " "	0.0233		97

analysis an aliquot is then diluted so that 1 ml. contains 0.005 to 0.01 γ of thiamine. Extracts prepared in this manner are usually clear and colorless. For slightly turbid or colored extracts an uninoculated tube containing the largest amount of the diluted sample can be used to obtain a blank correction for the "top" tube. Simple division of this optical density gives blank correction values for the other tubes of the sample.

Table II contains the values and recoveries of added thiamine for three widely different samples. There is good agreement at the four levels assayed and good recoveries of the added vitamin. The addition of 0.01 γ of

thiamine hydrochloride in a similar manner to twelve other biological materials has given recoveries of 101 ± 8 per cent.

The values obtained for some materials are the same whether the sample is enzymatically digested or steamed with 0.1 N H_2SO_4 for 30 minutes. In others the extraction is not complete without enzymes. The thiamine in dehydrated foods, yeasts, etc., is particularly difficult to extract completely. Samples of this nature are therefore steamed with 0.1 N H_2SO_4 for 30 minutes, neutralized to pH 4.5, and digested with enzymes as above.

TABLE III

Comparative Thiamine Assays

Material	Lactobacillus fermentum	Other methods (units per gm)	
	γ per gm	γ	
Cereal Product 1	6.9	7.0	Thiochrome*
" " 2	10.3	10.5	" *
Dehydrated pork	28.0	28.6	" *
Dried yeast	640	700	Yeast fermentation and thiochrome†
White flour, unenriched	0 74	0.78	Thiochrome‡
Toasted cereal	1.89	1.65	" ‡
Wheat germ extract	131	137	" §
		160	Melnick-Field§
	mg. per gm.	mg	
Dried yeast "	0.92	0.94	Thiochrome§
Fortified liver "	6.2	8.1	Melnick-Field§

We are indebted for the above samples and analyses by other methods to (*) Dr· C. A. Elvehjem, University of Wisconsin, (†) Dr. C N Frey, The Fleischmann Laboratories, (‡) Dr. J S. Andrews, General Mills, Inc , and (§) Dr A. D. Emmett, Parke, Davis and Company.

Values for urines are almost the same whether enzymatically digested or merely analyzed after the proper dilution. In general recoveries of added thiamine are more consistent if the samples are digested first

Results

In Table III thiamine values obtained with *Lactobacillus fermentum* are compared to those obtained with other assay methods. Samples which had been analyzed by yeast fermentation, thiochrome, and chemical methods in other laboratories were kindly given to us for analysis by *Lactobacillus fermentum*. In most cases the values agree closely. The values by the Melnick-Field method (6) appear high compared both to thiochrome and to *Lactobacillus fermentum*.

The thiamine values for some foods, tissues, etc., which have been

analyzed by use of *Lactobacillus fermentum* are shown in Table IV. These values are in general agreement with figures in the literature for similar samples. Recoveries of thiamine added to some of these materials were previously shown in Table II.

TABLE IV

Thiamine Assays by Lactobacillus fermentum

Material	Thiamine per gm.	Material	Thiamine per gm.
	γ		γ
Rat liver	7.3	White bread, unenriched	0.47
" muscle	1.5	" rolls, enriched	1.85
Hamster liver	3.5	Egg	0.75, 0.79
" muscle	1.0	Ham	7.5, 7.6
Orange juice*	0.9	Sausage, pork	6.4, 5.5
Tomato "	0.35	Veal loaf	1.0
Corn	0.23	Hamburger	0.3
Potatoes	0.5, 0.6		

* Orange juice and the other foods are samples obtained from restaurants

SUMMARY

A microbiological assay procedure for thiamine is proposed, based on the growth response of *Lactobacillus fermentum* to thiamine. In 16 to 18 hours heavy growth is obtained with 0.005 to 0.04 γ of thiamine, but there is no response to similar amounts of the "pyrimidine" and "thiazole" halves of the thiamine molecule either alone or together or in the presence of thiamine or biological extracts. Cocarboxylase is about 30 per cent more active than equimolecular amounts of thiamine.

For assay purposes enzymatic digestion of all samples is recommended for complete extraction and conversion of cocarboxylase to thiamine.

Good agreement of thiamine values of samples assayed at different levels and good recoveries of added thiamine have been obtained for many biological materials including foods, animal tissues, and urine.

BIBLIOGRAPHY

1. Schultz, A. S., Atkin, L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.,* **14,** 35 (1942).
2. Williams, R. J., McMahan, J. R., and Eakin, R. E., *Univ Texas Pub., No. 4137,* 31 (1941).
3. Schopfer, W. H., and Jung, A., *Compt. rend. Acad.,* **204,** 1500 (1937).
4. Hamner, K. C., Stewart, W. S., and Matrone, G., *Food Res.,* **8,** 44 (1943).
5. Niven, C. F., Jr., and Smiley, K. L., *J. Biol. Chem.,* **150,** 1 (1943).
6. Melnick, D., and Field, H., Jr., *J. Biol. Chem.,* **127,** 515 (1939).

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7. Alexander, B., *J. Biol. Chem.*, **151,** 455 (1943).
8. Jansen, B. C. P., *Rec. trav. chim. Pays-Bas*, **55,** 1046 (1936).
9. Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, **61,** 179 (1939).
10. Mason, H. L., and Williams, R. D., *J. Biol. Chem.*, **140,** 417 (1941).
11. Stiles, H. R., Peterson, W. H , and Fred, E. B., *J. Biol. Chem.*, **64,** 643 (1925).
12. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal Ed.*, **11,** 346 (1939).
13. Stokes, J. L., and Martin, B. B., *J. Biol. Chem.*, **147,** 483 (1943).
14. Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135,** 213 (1940).
15. Cheldelin, V. H , Eppright, M A., Snell, E. E., and Guirard, B. M., *Univ. Texas Pub., No. 4237*, 15 (1942).

THE ALLANTOIN CONTENT OF BLOOD

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Although allantoin is considered to be the end-product of purine metabolism in most mammals except the higher Primates, there are few values in the literature for the allantoin content of the blood of such animals. If the values obtained by Hunter (1) for the allantoin content of beef and hog blood (0.78 and 0.58 mg. per 100 cc., respectively) by an isolation procedure are approximately correct, it is obvious that many of the earlier methods employed for urine analysis would not be sufficiently sensitive for the determination of allantoin in blood.

Fosse and coworkers (2–4) gave a new impetus to the study of allantoin methods by the application of the Rimini-Schryver color reaction for formaldehyde to glyoxylic acid which is formed by the hydrolysis of allantoin. The glyoxylic acid is coupled with phenylhydrazine and the hydrazone is oxidized in a strongly acid medium with potassium ferricyanide to produce a colored oxidation product, which is measured colorimetrically. Allantoin is hydrolyzed by the allantoinase of soy bean meal to allantoic acid, which by hydrolysis with acid gives urea and glyoxylic acid.

Young and Conway (5) have greatly simplified the procedure of Fosse and coworkers by the substitution of a short hydrolysis with weak alkali at 100° for the tedious enzyme hydrolysis. These workers have also reexamined the optimum conditions for the acid hydrolysis of the allantoic acid to glyoxylic acid and the oxidation of the phenylhydrazone of this compound. In agreement with Borsook (6) it was found that the oxidation should be carried out at low temperatures to obtain the best results. Young and Conway applied the method to the determination of allantoin in urine and were able to use the direct vision colorimeter for the colorimetric measurements. More recently (1944) Young and coworkers (7) have applied their procedure to the determination of allantoin in blood, using either a direct vision or a photoelectric colorimeter.

In 1942, Esterer (8) in this laboratory applied the general technique described above to the determination of allantoin in amounts ranging from 2.5 to 25 γ per 5 cc. of solution. This was made possible by the use of the Evelyn photoelectric colorimeter. Filter 520 was found to transmit light which was strongly absorbed by the faint pink color produced in the final step of the procedure. The time for the maximum color formation

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and the rate of fading varied somewhat from that noted by Young and Conway for higher concentrations of allantoin. To obtain accurate results it was necessary to set up a very carefully timed schedule, so that the colors could be read at a definite time interval after the addition of the ferricyanide.

Both Borsook (6) and Young and Conway (5) carried out the oxidation of the phenylhydrazone after cooling the tubes containing the glyoxylic acid and phenylhydrazine to −10°. In our hands it was found difficult to maintain this temperature exactly in different experiments. In some cases the contents of the tube froze during the cooling period, and thus delayed the time at which the ferricyanide was added. For this reason, the procedure was modified to the extent that the temperature was adjusted to 0° rather than to −10°. Although the color produced per unit of allantoin is slightly less by this modification of the method, it was also noted that the rate at which the color faded was not as rapid as that obtained at lower temperatures. Over a range of 2.5 to 25 γ of allantoin, the absorption of light by the colored oxidation product was in accordance with the Beer-Lambert law.

Rabbit blood, deproteinized with tungstic acid, was analyzed by the above procedure and values from 1 to 2 mg. of allantoin per 100 cc. of blood were obtained. Recoveries of added allantoin ranged from 99 to 102 per cent. It was noted, however, that following the alkaline hydrolysis the tungstic acid filtrates of some of the bloods had a faint brown color which was not obtained with pure allantoin solutions. Since this suggested the presence in the blood filtrate of some interfering substance or substances, a study of such compounds was made by Foster (9) during the summer of 1943. Young and Conway have recorded a number of substances which give interfering colors. Since their work was concerned with the allantoin content of urine, which could be diluted 25 to 50 times prior to the analysis, it was thought advisable to check the interference of some compounds, the content of which in proportion to the allantoin might be greater in blood than in urine. Glucose was one of the compounds listed by Young and Conway which did not interfere with the determination, although a synthetic urine which contained glucose gave a higher value for allantoin than a similar urine from which glucose was omitted. In the present work it was found that if 500 γ of glucose were carried through the procedure for allantoin, a color equivalent to 1 γ of allantoin was obtained. Since 5 cc. of the tungstic acid filtrate of most bloods will contain between 400 and 600 γ of glucose, and in most cases less than 10 γ of allantoin, the color contributed by glucose will be a considerable percentage of the total color.

Uric acid was found by Young and Conway to give approximately one-

eighth of the color given by an equivalent amount of allantoin. By our procedure, 1 mg. of uric acid gives approximately one-third of the color given by 1 mg. of allantoin, while ergothioneine, which is present in considerable amounts in some animal bloods, gives one-twentieth of the color given by allantoin. The analyses of solutions containing both allantoin and uric acid or allantoin and ergothioneine indicate that the colors which are produced are additive. It is possible therefore to correct for the presence of uric acid and ergothioneine in blood filtrates if the amounts of these substances are known.

Ascorbic acid, pyruvic acid, creatine, or creatinine, in the amounts which would be present in blood, does not interfere with the method.

Reagents—

1. 0.5 N sodium hydroxide and 0.5 N hydrochloric acid. Slight variation from the above normality is permissible provided the two solutions are equivalent.

2. Phenylhydrazine hydrochloride solution (0.33 per cent). 83 mg. of the salt which has been recrystallized from alcohol until it is colorless are dissolved in 25 cc. of distilled water. This solution should be colorless and may be used over a period of 5 to 6 hours if it is kept in an ice bath out of direct light.

3. Potassium ferricyanide (1.64 per cent). 416 mg. of the salt (reagent quality) are dissolved in 25 cc. of water. A fresh solution must be made each day.

4. 10 N hydrochloric acid.

5. 25 per cent suspension of yeast cells. Two or three yeast cakes (Fleischmann's bakers' yeast) are suspended in 200 cc. of distilled water and transferred to centrifuge tubes. After centrifugation the milky supernatant fluid is discarded and the cells resuspended in distilled water and again centrifuged. This process is repeated several times until the supernatant liquid is perfectly clear. For use, 1 gm. of washed cells is suspended in 3 cc. of distilled water. Enough yeast may be washed for 10 days work, but the suspension must be kept in the ice box and the cells rewashed daily, until the supernatant liquid is clear. Usually one or two washings are sufficient after the initial washing.

Method

Allantoin prepared by oxidation of uric acid was recrystallized several times. The final product had a melting point of 224°, nitrogen content of 35.3 per cent, and contained less than 0.1 per cent uric acid as an impurity. Solutions of this material containing 2.5 to 25 γ of allantoin per 5 cc. were used to establish the constant K used in the determinations of allantoin in tungstic acid filtrates of blood.

The steps in the procedure are as follows: (1) In each of four test-tubes (15 × 150 mm.), graduated at 13.5 cc., are placed 5 cc. of allantoin solution. 5 cc. of distilled water are placed in a fifth tube. (2) 1 cc. of 0.5 N NaOH is added to each tube and after mixing the tubes are immersed in a boiling water bath for exactly 7 minutes. (3) The tubes are then placed in a water bath at 18° for 3 minutes. (4) To each tube are added 1.3 cc. of 0.5 N HCl and 1 cc. of the phenylhydrazine solution. The contents of the tube are again mixed by rotation. (5) The tubes are now immersed in a boiling water bath for exactly 2 minutes and at the end of this time are immediately placed in an ice bath of sufficient size to insure the rapid cooling of the tubes to 0°. The tubes are allowed to remain in the bath for 5 to 10 minutes. The remainder of the procedure should be carried out in close proximity to the colorimeter in order that the color evaluation may be made at a definite interval after the addition of the potassium ferricyanide. (6) To the first tube (water blank) add 4 cc. of 10 N hydrochloric acid that has been previously chilled to 0°. The time at which the hydrochloric acid is added to the blank tube is recorded as zero time (preferably on a stop-watch). At the $\frac{1}{2}$ minute interval, the tube is removed from the ice bath and at the 1 minute interval 1 cc. of the ferricyanide solution is added to this tube. The volume of fluid in the tube is then adjusted to 13.5 cc. with distilled water and after mixing by inversion the tube is placed in a water bath at 20°. At the 2 minute interval, hydrochloric acid is added to the second tube and the process is repeated. The fifth tube of the series will thus receive the acid at the 8 minute interval and the ferricyanide at the 9 minute interval. As soon as the fifth tube can be diluted to volume (usually 10 minutes, counting from zero time), the contents of the first tube (water blank) are transferred to a dry, clean colorimeter tube. The tube is placed in the colorimeter (Filter 520) and the transmission adjusted to 100. When the blank tube is removed, the galvanometer reading usually falls to 73^2 or 73^3 (center setting). Since the light transmitted through the blank tube changes slightly during the 10 to 20 minute interval during which the remaining tubes are read in the colorimeter, it is advisable to readjust the colorimeter to the center setting if there is any tendency for the galvanometer reading to drift during this period. The remaining tubes are placed in the instrument and the maximum absorption recorded. Usually this value is obtained in the period of 8 to 11 minutes after the ferricyanide has been added.

By this procedure the K values for a series of concentrations of allantoin ranging from 2.5 to 25 γ per 5 cc. of solution were 0.0317 ± 0.0004. $K = L/C$, where $L = 2 - \log$ galvanometer reading, and $C =$ the micrograms of allantoin per 5 cc. of solution. The lowest K values were found for the weaker concentrations of allantoin.

Comments on Procedure

Since the volumes of the various reagents used in the method are relatively small, studies were made to determine whether slight variations in the measurement of these solutions would lead to a significant difference in the final color value. The results indicated that small differences in the volume of the 0.5 N alkali or acid did not affect the final color intensity. The color intensity can be measurably decreased or increased, however, if the volume of phenylhydrazine solution varies by more than 0.1 cc. from that recommended.

In the directions given by Young and Conway, 3 cc. of concentrated hydrochloric acid were recommended to provide the acid reaction suitable for the oxidation of the phenylhydrazone. In the course of our studies it was found that the color intensity from a given quantity of allantoin was greater if 3 cc. of concentrated hydrochloric acid containing 38 per cent acid were used as compared to 3 cc. of 35 per cent hydrochloric acid. To avoid this source of error, 4 cc. of 10 N hydrochloric acid are recommended in the present method. Although the final acidity produced by the addition of 4 cc. of 10 N hydrochloric acid is greater than that due to 3 cc. of concentrated hydrochloric acid (11 to 12 N), the color intensity from a given amount of allantoin can be further increased by the use of 4.5 or 5.0 cc. of the 10 N acid. The increase in color intensity is not sufficient to compensate for a more rapid rate of color development, followed by a more rapid rate of fading. The measurement of 4 cc. of 10 N hydrochloric acid should be accurate to within 0.1 cc.

It should also be noted that although the period of hydrolysis with alkali could be extended from 7 minutes to 8 minutes, and the period of acid hydrolysis increased from 2 to $2\frac{1}{2}$ minutes without a further increase in color intensity, a decrease in these hydrolysis periods of more than 15 seconds will lead to low results.

Determination of Allantoin in Blood

To 1 volume of blood are added 5 volumes of distilled water and 1 volume of washed yeast cells. Incubate at room temperature for 10 minutes. Water, 10 per cent sodium tungstate, and $\frac{2}{3}$ N sulfuric acid are now added to give a total of 10 volumes, and the solution is filtered. When 5 cc. of blood were used, 6 cc. of the 10 per cent sodium tungstate and 6 cc. of the $\frac{2}{3}$ N H_2SO_4 were used instead of 5 cc. portions of these reagents in order to insure the complete precipitation of the protein of both the blood and the yeast cells. 5 cc. portions of the tungstic acid filtrate are used for the analysis of allantoin as described above.

To determine whether the method of deproteinization and removal of glucose with yeast lead to a loss of allantoin, experiments were made with a

20 per cent protein solution prepared from dried egg albumin. 5 cc. portions of this solution were deproteinized with tungstic acid in the usual manner. The analysis of the resulting filtrate for allantoin gave a blank value equivalent to 1.6 γ of allantoin per 5 cc. of filtrate. Allantoin added to a second portion of the protein prior to deproteinization was quantitatively accounted for after the deduction of this blank. Incubation of additional portions of the protein solution with the yeast cells with or without the addition of allantoin indicated that the yeast cells did not add interfering materials to the filtrate or destroy any of the allantoin. Glucose added to 5 cc. of the protein solution in amounts 2 and 3 times as great as that found in an equal volume of blood was completely removed by 5 cc. of the yeast cell suspension during the 10 minutes incubation period at room temperature. Similar experiments made with animal bloods indicated that the removal of glucose by treatment with yeast did not interfere with the determination of allantoin and that allantoin added to such bloods could be quantitatively recovered. If one wished to assume that most bloods contain approximately 100 mg. of glucose per 100 cc., a correction factor of 0.2 mg. of allantoin per 100 cc. of blood could be deducted and thus eliminate the treatment with yeast cells. As will be seen from Table I, such a correction would represent a large percentage of the total allantoin of some bloods.

Since uric acid and ergothioneine interfere with the method for allantoin, a procedure for deproteinization which would remove these substances without loss of allantoin would be desirable. Deproteinization by tungstic acid does not remove uric acid and ergothioneine, but these latter substances can be removed when blood is deproteinized with alkaline solutions of zinc, copper, cadmium, or iron salts. Unfortunately, all of these alkaline precipitating reagents proved unsatisfactory, since either part of the allantoin was lost or undesirable turbidities appeared at some step in the subsequent allantoin analysis.

Since these difficulties could not be overcome, the deproteinization by tungstic acid was adopted, and the total "allantoin color" obtained on a 5 cc. portion of this filtrate. Corrections are then applied for the color contributed by ergothioneine and uric acid. It will be noted in Table I that in some bloods these corrections are relatively large and in other bloods relatively small. Uric acid was determined by the isolation procedure of Folin (10), and ergothioneine by the method of Behre and Benedict (11).

Since the present study was not concerned primarily with the ergothioneine content of blood but with the ergothioneine content of the tungstic acid filtrate of blood, molybdic acid as the protein precipitant, as recommended by Benedict, was not used. The ergothioneine values in the paper

must therefore be considered as minimal if Benedict's observation that part of the ergothioneine is lost by tungstic acid deproteinization (11) is correct.

Table II illustrates the recovery of allantoin added to blood of various animals. In every case the allantoin was added prior to deproteinization in such an amount that 5 cc. of the tungstic acid filtrate would contain 10 γ of added allantoin. The original allantoin content given in the second column would include uric acid and ergothioneine as well as allan-

TABLE I

Allantoin Content of Various Bloods

All values are expressed as mg. per 100 cc. of blood. The figures in parentheses give the range of values for individual animals.

Animal*	No. of animals	Total allantoin color	Uric acid	Ergothioneine	Corrected allantoin†
		mg	*mg.*	*mg*	*mg.*
Cow	6	2.93 (2 74–3 27)	0.79 (0 61–0 87)	1.2	2.61 (2.39–2.93)
Calf	6	2 39 (1.83–3 53)	0.66 (0.52–0.87)	1.2	2.11 (1.59–3.22)
Sheep	5	2.07 (1 52–2 51)	0 22 (0 20–0 24)	3.8 (2 5– 5 6)	1.80 (1.25–2.31)
Rabbit‡	5	1 79 (1 29–2 17)	0 23 (0 18–0.28)	8.7 (6 1–10 0)	1.28 (0.93–1.71)
" §	2	3 00 (2 67–3 32)	0.22 (0 19–0 24)	3 0 (2.9– 3.1)	2.77 (2 44–3.10)
Dog	6	1.58 (1.35–1 81)	0.24 (0 19–0 38)	13 8 (8.3–18 2)	0.81 (0.38–1.30)
Rat‖	6	2 07 (1.63–2 48)	2 40 (1 5 –3.20)	2.0	1.15 (0 89–1.67)
" ¶	7	1.91 (1 45–2 26)	1 6 (1 3 –2 2)	2 0	1.28 (0.85–1.65)
Hog	7	0 76 (0.63–1 01)	0.16 (0.12–0.19)	7 6 (5.9–11.2)	0.33 (0.06–0.62)

* For analyses of horse, chicken, and human blood, consult the text.

† The sum of one-third of the uric acid content plus one-twentieth of the ergothioneine content has been subtracted from the values in the third column, to give the figures in this column.

‡ These were albino rabbits.

§ These rabbits were white and black   Both groups of rabbits were fed *ad libitum* on the same diet   A second analysis on the blood of the black and white rabbits gave values of 4.37 and 3 24 mg of allantoin (corrected) per 100 cc. of blood.

‖ Rats in this group were on a high protein diet (30 per cent casein).

¶ Rats in this group were on a low protein diet (5 per cent casein).

toin for most of the bloods. In the case of the chicken and human blood the value given is probably due entirely to substances other than allantoin. In Table I this value is given in the third column as "total allantoin" color.

The recoveries of added allantoin range from 98 to 106 per cent, with an average value of 102 per cent. If the recoveries from chicken and human blood are omitted, the average recovery is approximately 101 per cent.

It will be noted in Table II that allantoin added to horse, chicken, and

human blood could be quantitatively recovered, but allantoin values for these bloods do not appear in Table I. It was not expected that human or chicken blood would contain allantoin but it was somewhat surprising to find that after the total allantoin value for whole horse blood was corrected for ergothioneine and uric acid a negative value for allantoin was obtained. The total allantoin (range, 0.43 to 0.84 mg.; average, 0.59 mg.) for the horse blood is lower than for any of the animals recorded in Table I. Since the average uric acid of horse blood was 0.35 mg. per 100 cc., the correction for this constituent was relatively small. The ergothioneine

TABLE II

Recovery of 10 γ of Allantoin Added to Whole Blood

All values are expressed as micrograms per 5 cc of tungstic acid filtrate.

Animal	Original	Total	Recovery
			per cent
Cow 1	13.8	24.2	104
" 2 .	14.8	24 9	101
Sheep 1	7.6	17.9	103
" 2	10 0	20 0	100
Rabbit 1	6.5	16.6	101
" 2	10.6	20.9	103
Dog 1 .	6.8	16.6	98
" 2...	9.0	19.0	100
Horse 1*	2.4	12.6	102
" 2 .. .	2.2	12.3	101
Chicken 1* .	3.0	13 3	103
" 2 .	3.3	13.7	104
Man 1*	4.2	14 8	106
" 2	4.2	14.6	104

* The greater part of the color obtained by the method when horse blood is analyzed probably is due to the presence of uric acid and ergothioneine. All of the color obtained from human and chicken blood can be explained by their content of these two substances.

values, however, are the highest obtained for any species of blood, ranging from 12 to 21 mg. per 100 cc. It seems possible that some substance other than ergothioneine is included in this fraction and thus a correction for allantoin is being applied that is too high. Evidence for this view was obtained by the analysis of horse blood plasma. The plasmas of two of the six horse bloods were found to contain 0.69 and 0.87 mg. of allantoin per 100 cc., after a correction was applied for the small amount of known interfering materials contained in the plasma. Fosse and coworkers (12) reported 1.28 mg. of allantoin per 100 cc. of horse serum.

The total allantoin color obtained in human and chicken blood can in

most cases be accounted for by the content of uric acid. These bloods also contain a small amount of ergothioneine and, after the correction for this substance is added to that for uric acid, a negative value for allantoin is obtained. The reason for these negative results is „unexplained. It is possible that the uric acid values are high or that the interference of uric acid and ergothioneine is not quantitatively the same in a blood filtrate as in pure solution.

In the analyses recorded in Table I, the correction for uric acid is relatively unimportant except in the case of beef and rat blood. Benedict and coworkers (13, 14) have shown that beef blood contains combined uric acid as well as some free uric acid. The combined uric acid is not precipitated with ammoniacal magnesia mixture, but no data are available concerning its precipitation by the acid silver lactate reagent of Folin. Some comment may be made on the high uric acid content of rat blood. Blauch and Koch (15) have pointed out that the uric acid content of freshly drawn rat blood is low but rapidly increases on standing. The tungstic acid filtrates in the present work were prepared immediately after a group of rats (six or seven) were killed. Some of the bloods were probably not deproteinized for 1 to 1½ hours after withdrawal, while others were deproteinized within 30 minutes. The level of uric acid found, however, does not closely parallel the periods of standing at laboratory temperature. Regardless of the uric acid content, the corrected allantoin values are as consistent as those for any other animal studied. The allantoin of the blood of rats on a high protein diet was essentially the same as for those on a low protein diet.

Little comment is necessary concerning the results recorded in Table I. For the herbivorous animals, the level of allantoin in blood is only slightly lower than the uric acid level of human blood, if the uric acid analyses are made by methods which give the true uric acid. The only allantoin figures recorded in the literature to which these results can be compared are those of Young and coworkers (7), and Fosse and coworkers (12). The former found from 0.82 to 1.11 mg. of allantoin per 100 cc. of dog blood and somewhat less than half of this amount in the blood of the Dalmatian coach hound. The blood of three cats contained 1.09, 2.4, and 3.17 mg. of allantoin per 100 cc. The values for dog blood are somewhat higher than our corrected values. The blood of one cat was analyzed in the present study, before the interference of uric acid, glucose, and ergothioneine was appreciated, and a value of 2.0 mg. per 100 cc. of blood was obtained. Unfortunately, cats were not available when the later studies were made.

Fosse and coworkers (12) analyzed the serum rather than the whole blood of various animals and the results expressed as mg. per 100 cc. of serum, with the number of animals given in parentheses, are as follows:

cows (six) 1.07 to 2.22, calves (five) 1.54 to 2.65, horse (one) 1.28, sheep (one) 1.92, hogs (three) 1.24 to 1.83. The values for cattle and sheep agree fairly well with corrected values for the whole blood of these animals. The hog serum values are distinctly higher than those obtained by us on whole blood. The two horse plasmas which were analyzed by us have a somewhat lower content of allantoin than the 1.28 mg. reported in Fosse's work.

Since it appears that the work on this problem must be suspended for an indefinite period, several questions of interest must remain unanswered. The higher levels of allantoin noted in the blood of two black and white rabbits (Table I) as compared with pure albino rabbits should receive further study. It would also be of interest to study the distribution of allantoin between the plasma and red blood cells of the various species of animals. A method which would permit the quantitative separation of allantoin from other blood constituents prior to the analysis would be desirable. Preliminary attempts to remove allantoin as the mercuric salt from tungstic acid filtrates gave values for allantoin which were from 60 to 70 per cent of the corrected values given in the present paper.

SUMMARY

A method for the determination of allantoin in blood is described. The method depends upon the formation of glyoxylic acid by the hydrolysis of allantoin. The color obtained by the oxidation of the glyoxylic acid phenylhydrazone with ferricyanide in strongly acid medium is estimated in a photoelectric colorimeter.

Glucose, uric acid, and ergothioneine are constituents of blood which are known to interfere with the method. Glucose is removed by yeast treatment prior to deproteinization, and corrections are applied for the presence of uric acid and ergothioneine.

Values for the allantoin content of 50 blood samples which include beef, sheep, rabbit, dog, rat, and hog blood are presented. Of this group the lowest content of allantoin was found in hog blood (0.33 mg. per 100 cc.), while the highest content was consistently found in adult beef blood (2.61 mg. per 100 cc. of blood).

BIBLIOGRAPHY

1. Hunter, A., *J. Biol. Chem.*, **28**, 369 (1916–17).
2. Fosse, R., and Hieulle, A., *Compt. rend. Acad.*, **179**, 636 (1924).
3. Fosse, R., Brunel, A., de Graeve, P., Thomas, P.-E₄, and Sarazen, J., *Compt. rend. Acad.*, **191**, 1388 (1930).
4. Fosse, R., Brunel, A., and Thomas, P.-E., *Compt. rend. Acad.*, **192**, 1615 (1931).
5. Young, E. G., and Conway, C. F., *J. Biol. Chem.*, **142**, 839 (1942).
6. Borsook, H., *J. Biol. Chem.*, **110**, 481 (1935).
7. Young, E. G., MacPherson, C. C., Wentworth, H. P., and Hawkins, W. W., *J. Biol. Chem.*, **152**, 245 (1944).
8. Esterer, M. B., Thesis, University of Michigan (1942).
9. Foster, P. W., Thesis, University of Michigan (1943).
10. Folin, O., *J. Biol. Chem.*, **101**, 111 (1933).
11. Behre, J. A., and Benedict, S. R., *J. Biol. Chem*, **82**, 11 (1929)
12. Fosse, R., Brunel, A., and Thomas, P.-E., *Compt. rend. Acad.*, **193**, 7 (1931).
13. Benedict, S. R., *J. Biol. Chem.*, **20**, 633 (1915)
14. Davis, A. R., Newton, E. B., and Benedict, S. R., *J. Biol. Chem.*, **54**, 595 (1922).
15. Blauch, M. B., and Koch, F. C, *J Biol. Chem.*, **130**, 455 (1939).

THE MEASUREMENT OF ARGINASE ACTIVITY

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The chief object of this paper is to present certain improvements in Hunter and Dauphinee's method (1) for the measurement of arginase activity. Incidentally we describe a procedure for the concentration and partial purification of arginase, and report some observations upon its activation by cobalt.

In principle the method of Hunter and Dauphinee consisted in determining the amount of urea produced by a known volume of the arginase-containing solution acting, for a given time and under certain defined conditions, upon a known quantity of arginine, and referring this to a standard curve showing the empirically ascertained relation between urea production (under the chosen conditions) and quantity of enzyme. The same principle had been used already by Edlbacher and Röthler (2).

Continued use of the method has revealed certain defects in the original prescription. (*a*) No care was taken to provide that, whether in the preparation of the reference curve or in the testing of an unknown, the volume of the enzyme-substrate mixture (and therefore the concentration of the arginine) should be always the same. (*b*) The action of the enzyme was measured in a phosphate mixture at a pH (8.4) at which the buffering effect of phosphate is very slight. (*c*) The unit of arginase activity was defined from a point of the reference curve so near the origin that its exact location was largely guesswork. (*d*) The crude jack bean extract, employed as a source of urease, contained itself, as subsequently discovered by Hellerman and Perkins (3, 4), enough arginase to introduce an appreciable error into the determination.

Revising the method in the light of these considerations we have (*a*) adopted for all arginine-buffer-arginase mixtures a uniform volume of 8 ml., with a final arginine concentration of 0.02225 M, (*b*) substituted for phosphate a phosphate-phenolsulfonate mixture of the same pH and the same total buffer concentration, (*c*) redefined our arginase unit, and (*d*) replaced the excess of crude jack bean extract originally employed by an adequate but not unnecessarily large amount of a partially purified urease. In addition we have thought it advisable (*e*) to purify to some extent the arginase used in the construction of the standard curve and (*f*) to activate it fully with cobalt.

Buffer Solution—The buffer solution now used has the following composition: 0.5 M sodium *p*-phenolsulfonate 50 ml., M KH₂PO₄ 25 ml., 2 N NaOH 15.6 ml., water to 100 ml. This solution is 0.5 M in total buffer concentration, and has a pH of 8.4.

Unit of Arginase Activity—The unit of arginase activity we now define as one-tenth of that amount of arginase, which, under standard conditions (as defined later), liberates urea equivalent to 2.5 mg. of nitrogen. Upon the curve relating enzyme quantity to action (Fig. 1) the accurate location of the 2.5 mg. point presents no difficulty. On the original curve of Hunter and Dauphinee (1) this point, as it happens, corresponds to exactly 10 units of arginase. We have satisfied ourselves that the change in composition of the buffer is without appreciable effect upon the activity of the enzyme. The new and more precise method of defining the unit leaves its magnitude, therefore, unaltered.

Preparation of Suitable Urease Solution—The preparation of a urease absolutely devoid of argininolytic properties appears to be a difficult matter, but the following procedure will usually give a product suitable enough for the purpose in view. Jack bean meal, 100 gm., is extracted with 500 ml. of water, and the centrifuged extract is treated with 8 volumes of acetone. The precipitate is collected by centrifugation, dried over H₂SO₄, and ground to a fine powder. This is again extracted, this time with 400 ml. of water, and the processes of precipitation, drying, and grinding are repeated, to yield finally 20 to 25 gm. of powder. For use 0.5 gm. of this powder is dissolved in 100 ml. of 60 per cent glycerol. 1 ml. of this solution should decompose in 1 hour at room temperature urea equivalent to 9 to 10 mg. of nitrogen. Coincidental action upon arginine should be negligible. To test the latter point we determine the amount of ammonia nitrogen liberated during 1 hour at room temperature by the action of 1 ml. of the urease solution upon a mixture of 2 ml. of buffer solution, 5 ml. of 3 per cent arginine hydrochloride solution, and 1 ml. of 0.04 per cent cobalt nitrate. Although this mixture contains 4 times the standard amount of arginine, the yield of ammonia nitrogen ought not to exceed 0.02 mg. By way of contrast it may be reported that 1 ml. of a crude urease solution, prepared according to the directions of Schmidt (5), has been found to yield under similar conditions as much as 0.4 mg.

Preparation of Partly Purified Arginase Concentrate—The enzyme solution used in preparing the original standard curve of reference was a suitably diluted crude liver extract. We have replaced this by a solution containing a smaller proportion of inert material, and, with other purposes in view, we find it convenient to combine partial purification with concentration. A fresh baby beef liver is drained as completely as possible of blood, and minced. 1 kilo of the moist product is stirred for 10 minutes with 1 liter

of water. The mixture is transferred to a flask, submerged in a bath of water at 65°, and stirred gently until the mass reaches a uniform temperature of 58°. After being maintained at this temperature for 5 minutes, the mixture is rapidly cooled and centrifuged. A measured volume, say 800 ml., of the supernatant is treated with 1.2 volumes of acetone. The resulting flocculent precipitate is separated as quickly as possible by centrifugation, and drained of adherent liquid. It is then taken up in 400 ml. (0.5 volume) of water, and stirred or shaken until as much as possible has gone into solution. Undissolved material is removed by centrifugation, and the clear supernatant is mixed with 1.2 times its volume of acetone. Again the mixture is rapidly centrifuged, the second acetone precipitate is treated like the first, and its clarified solution is subjected to a third precipitation. The third precipitate is centrifuged off and transferred to a vacuum desiccator. The desiccator is evacuated step by step, until all the acetone and most of the water have been evaporated. The residual waxy paste is then ground up thoroughly with 75 per cent glycerol in one-tenth of the initial volume (80 ml.). The turbid reddish brown solution thus obtained will contain at least half of the arginase originally present, so that the concentration of the enzyme will have been increased 5 times or more. Different preparations have contained, after full activation by cobalt, from 500 to 900 or more units per ml. The degree of purification attainable may be exemplified by one instance, in which the number of arginase units per mg. of N rose from 21 in the crude liver extract up to 73 in the final product.

A concentrate prepared in the way described and preserved at 10° will retain its original activity almost unimpaired for a year. Diluted with water it forms a milky suspension from which there gradually separates a more or less copious flocculent precipitate of water-insoluble protein. This precipitate carries down with it a part of the enzyme, so that the clear supernatant is only 85 to 90 per cent as active as the whole suspension. On the other hand the supernatant has a slightly higher ratio of arginase to nitrogen.

Activation of Arginase Solutions—The activity of arginase solutions or suspensions prepared from liver is increased by treatment with certain metals, such as manganese (6) or cobalt (3, 4, 7). This activation, like that of intestinal peptidase by manganese (8), is a time reaction. This is shown by such experiments as the two recorded in Table I.

In Experiment 1, 0.3 ml. of an arginase concentrate was mixed with 14 ml. of water at 37°. The dilute suspension was divided into two equal portions, A and B, each of which was thenceforward kept at 37°. To Portion B was added 0.05 ml. of a 2 per cent solution of anhydrous cobaltous nitrate. As soon thereafter as possible the first activity determina-

tions were started with 1 ml. portions, first of Portion A and then of Portion B. Other determinations were commenced 10, 20, 60, and 180 minutes later. The technique of these determinations was that prescribed later, and involved an action period of 30 minutes.

Experiment 2 differed in that the original diluted suspension (not the same as that of Experiment 1) was centrifuged, and only the supernatant solution was treated with cobalt. Such treatment, it may be noted, leads to the gradual formation, in the originally clear mixture, of a fairly bulky precipitate. A part of the total active enzyme is adsorbed on, or otherwise incorporated in, this precipitate. The activities recorded in the present experiments are those of the whole mixture, precipitate included.

TABLE I

Influence of Time on Activation of Arginase by Cobalt at 37°

Experiment No	Nature of arginase preparation	Time at 37° before activity determination	Portion A, without cobalt			Portion B, with cobalt		
			Urea N found	Activity indicated	Apparent change in activity	Urea N found	Activity indicated	Apparent change in activity
		min	*mg*	*units per ml*	*per cent*	*mg*	*units per ml*	*per cent*
1	Suspension	3	2.033	7 1		2.424	9.4	+32.4
		13	2 033	7.1	0.0	2.568	10.4	+46.4
		23	1.996	6.9	−2 8	2.704	11.4	+60.5
		63	1.976	6 8	−4 3	2.874	12.85	+81.0
		183	1.866	6 2	−12.7	3.013	14.1	+98 5
2	Supernatant	3	2 063	7 3		2.369	9 1	+24 7
		18	2.026	7.05	−3.4	2 623	10 8	+48.0
		63	1.967	6 8	−6.8	2.818	12 4	+69.8
		183	1.902	6.4	−12.4	3.011	14.1	+93.0

The time intervals, as given in Table I, are reckoned from the instant at which cobalt was added to Portion B of the enzyme solution. They define only the moments of sampling. The measured activities cannot be taken as the actual activities at these precise moments, for it can hardly be doubted that activation (or inactivation), unless already maximal, progressed appreciably during the 30 minutes required for each determination. This inherent ambiguity does not affect the general conclusions to be drawn from the data, (1) that in the absence of cobalt the diluted enzyme is gradually destroyed, and (2) that in the presence of the metal its activity progressively increases. Independent observations of similar import, but with manganese as activator, have been made by Archibald[1] at the Hospital of the Rockefeller Institute. Dr. Archibald found that at 50° maximal activation is attained in as little as 20 minutes. This we have since ascertained to be true for cobalt activation also. Evidence to that effect will

[1] Archibald, R. M., private communication.

be found in Table II, which incorporates the results of various experiments in which different dilutions of one arginase concentrate (the same as that of Table I) were exposed to the action of cobalt for various times at 20°, 37°, or 50°. In these experiments (the first with Preparation 4 excepted) the concentration of cobalt was 2.5 times greater than in the previous ones. From the results it appears that at 20° maximal activation requires more than 48 hours, at 37° more than 3 but not more than 24 hours, and at 50° not more than 20 minutes. It is also shown that the activated enzyme, in

TABLE II

Cobalt Activation of Arginase at Various Temperatures

Temperature	Diluted arginase preparation		Time with cobalt at given temperature	Activity		Increase of activity
	Preparation No	Nature		Initial without cobalt	With cobalt	
°C			hrs	units per ml	units per ml	per cent
20	3	Supernatant	24	6 8	12.6	85
	3	"	48	6 8	13.3	96
37	3	"	3	6.8	14.0	106
	4	Suspension	3	7.3	15.0	105
	4	"	3	7.3	15.4	111
	3	Supernatant	24	6.8	14.9	119
			min.			
50	3	"	20	6.8	15.1	122
	3	"	20*	6 8	15.3	125
	5	"	20	7.35	16.3	122
	5	Suspension	20	8.25	18.5	124
	6	Supernatant	20	7.1	15.7	121
	7	"	20	6.75	15.0	122
	7	"	30	6.75	15.0	122
	7	"	40	6.75	14.9	121

* Followed by 24 hours at 37°.

contrast with the unactivated, is very stable, withstanding without loss exposure to 50° for 20 minutes more or to 37° for 24 hours.

We have mentioned already that, when a clear centrifuged solution of arginase is treated with cobalt, a part of the enzyme is precipitated in active form. At 50° this part amounts, we find, to 20 to 30 per cent of the total activated enzyme. Thus, for example, 5 ml. portions of the three cobalt-treated mixtures which appear last in Table II (with total activities of 15.0, 15.0, and 14.9 units per ml.) were centrifuged, and the precipitate in each case (not wholly free from adherent liquid) was redispersed in 5 ml. of 0.05 per cent cobalt nitrate. The activities of the supernatants were then found to be respectively 11.1, 11.15, and 11.0 units per ml., those

of the precipitates 4.6, 4.05, 4.3 units per ml.   It will be seen that the whole activity was recovered in the sum of the two fractions, and that, in this instance, the proportion remaining in solution was 74 per cent.   In other instances it has been as high as 80 and as low as 71 per cent.

Preparation of Diluted and Activated Arginase Solution—For the construction of the standard reference curve one requires an arginase solution containing from 16 to 20 units of enzyme per ml.; that is to say, a solution, of which 1 ml., acting under standard conditions (as defined later) upon a standard amount of arginine, will liberate between 3.25 and 3.5 mg. of urea nitrogen.   Such a solution is obtained by appropriate dilution of any glycerol concentrate.   If the potency of the latter and its behavior upon activation are unknown, the dilution required, which may vary between 15- and 40-fold, will have to be ascertained by trial.   The process of dilution is conveniently combined with that of activation.   For the latter we have adopted the conditions recommended by Archibald.[1]   The procedure is accordingly as follows:

1 ml. of the concentrate is mixed with the required amount of water. A 5 per cent solution of anhydrous cobalt nitrate is added at the rate of 0.06 ml. for each 10 ml. of the mixture.   This is enough to insure maximum activation and an optimum concentration of cobalt in the final digest. The mixture is now set in a water bath at 50° and left there for 20 minutes. The flocculent precipitate, which separates, is centrifuged off, and only the clear, practically colorless supernatant is made use of.   This may be only 60 to 70 per cent as active as the uncentrifuged mixture, for the centrifuge removes not only the originally suspended arginase (10 to 15 per cent of the whole) but also that precipitated by cobalt (20 to 30 per cent of the remainder).   The homogeneity of the product is an obvious counterbalancing advantage.   It may be added that centrifugation removes not only active enzyme but also some inert nitrogenous material, for in the final supernatant the ratio of arginase to nitrogen is greater than in the original concentrate.   In one instance it rose from 52 to 64 units per mg. of nitrogen, and in another it even reached 100.

Construction of Standard Reference Curve

Reagents—

Dilute arginase solution, as described in the preceding section.

Arginine hydrochloride, 1.875 per cent solution in the 0.5 M phosphate-phenolsulfonate buffer mixture of pH 8.4 already described.   2 ml. of this solution contain 9.975 mg. of nitrogen and will yield therefore a maximum of almost 5 mg. of urea nitrogen.

Urease solution, 0.5 per cent in 60 per cent glycerol.   The preparation of, and specifications for, this solution have been given earlier.

Hydrochloric acid and sodium hydroxide solutions, each about 2 N.

Phenol red indicator solution, 0.05 per cent.

Standard acid, standard alkali, and indicator as required for the micro-titrimetric determination of ammonia.

Procedure—Into each of ten Van Slyke-Cullen urea tubes are measured 2 ml. of the buffered arginine solution. To these tubes, numbered consecutively, there are further added 5.9, 5.8, ... 5.1, and 5.0 ml. of water. Into an ordinary test-tube are put a few ml. of the arginase solution. All the tubes are then set in a thermostat at 37°.

When the tubes have reached the temperature of the bath, 0.1 ml. of the arginase solution is transferred to Tube 1, the contents are rapidly mixed, and the time is noted. To Tubes 2 to 10 there are added in the same way at exactly measured convenient intervals 0.2, 0.3, ... 0.9, and 1.0 ml. respectively of arginase. It will be remarked that the total volume of each completed mixture is the same, namely 8 ml. The concentration of arginine in each is 0.02225 M.

To each tube in turn, about 29 minutes after the addition of the arginase, there is added a drop of phenol red indicator. Exactly upon the 30th minute the tube is removed from the thermostat, and treated with as many drops of 2 N HCl as are required to turn the indicator bright yellow. The action of the arginase having been thus inhibited, the contents of the tube are boiled, until the protein present has been completely coagulated.

The tubes having been cooled, each is treated drop by drop with 2 N NaOH, sufficient to give a just perceptibly pink color (pH about 6.8). To each is added finally 1 ml. of the urease solution. This is allowed to act at room temperature for 1 hour, and the urea determinations are completed in the usual way. The results are to be corrected by a control, in which a mixture of 2 ml. of arginine-buffer solution and 6 ml. of water, adjusted to a pH of about 6.8, is treated with urease in the same way as the principal mixtures.

A graphical representation of the results enables one to estimate the volume of arginase solution, which would give exactly 2.5 mg. of urea nitrogen. This volume contains, by definition, 10 units of the enzyme. On this basis the ml. of arginase solution are translated into units of arginase. The final reference curve is plotted accordingly, with mg. of urea nitrogen as ordinates and arginase units as abscissae. The curve thus obtained is reproduced in Fig. 1. In spite of the many extra precautions taken in its preparation, it differs but little from the original curve of Hunter and Dauphinee.

In the interest of accuracy it is best to restrict the use of the curve to the part between 1.25 and 3 mg. of nitrogen (3.5 to 14 units) of arginase. With higher yields of nitrogen the flattening out of the curve increases the significance in arginase units of any given error in the urea determination.

With lower yields the same absolute error becomes an unduly large fraction of the true value.

Measurement of Arginase Activity—The reagents required are those listed in the preceding section, with the exception, of course, of the dilute arginase solution. The place of the latter is taken by the solution of which the activity is to be determined. This will usually have to be diluted. For fresh liver extracts a 10-fold dilution will generally be about right; for concentrates a 20-fold or even a 100-fold dilution may be necessary. The correct proportion can be ascertained only by trial.

The procedure is as follows: Into one urea tube are measured 2 ml. of the arginine-buffer solution; into another, serving as a control, 2 ml. of plain
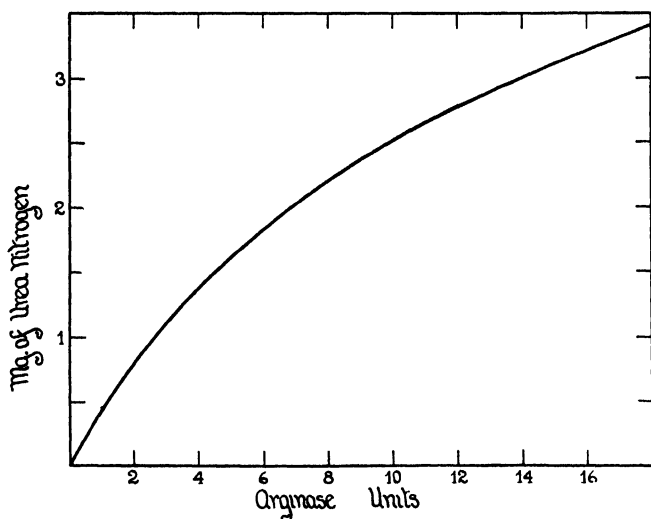
Fig 1. Standard reference curve for measurement of arginase activity

buffer solution. To each there are added 5 ml. of water. Both tubes are then set in the thermostat at 37°, along with a test-tube containing some of the solution of which the arginase activity is to be tested.

As soon as it is certain that the tubes have attained the temperature of the bath, 1 ml. of the enzyme solution is transferred (*a*) to the arginine tube and (*b*) to the control. The time of each addition is noted. From this point both tubes are treated in the manner described in the preceding section, so that the contents of each are acidified and boiled exactly 30 minutes after the addition of the enzyme, then neutralised, and submitted to a urea determination. The main result, corrected by the control, should fall between 1.25 and 3 mg. of urea nitrogen. If it is outside these

limits the process should be repeated with a more appropriate dilution of the original enzyme solution. When an acceptable result is obtained, it is referred to the standard curve, from which one may read directly the number of arginase units in the 1 ml. of diluted enzyme solution used.

SUMMARY

Details are given of an improved method for the measurement of arginase activity.

A procedure is described for the concentration and partial purification of liver arginase.

The activation of arginase by cobalt is shown to be a time reaction.

BIBLIOGRAPHY

1. Hunter, A., and Dauphinee, J. A , *J. Biol. Chem.*, **85,** 627 (1929–30).
2 Edlbacher, S., and Rothler, H., *Z. physiol. Chem.,* **145,** 69 (1925).
3 Hellerman, L., and Perkins, M. E., *J. Biol. Chem.*, **112,** 175 (1935–36).
4 Stock, C. C , Perkins, M. E., and Hellerman, L , *J. Biol. Chem.*, **125,** 753 (1938).
5 Schmidt, E. G., *J. Biol. Chem.*, **78,** 53 (1928).
6. Klein, G , and Ziese, W., *Klin. Wochschr* , **14,** 205 (1935).
7 Hellerman, L., and Stock, C C , *J. Biol. Chem* , **125,** 771 (1938).
8 Smith, E , and Bergmann, M., *J. Biol. Chem.*, **138,** 789 (1941).

THE DIRECT DETERMINATION OF VALINE AND LEUCINE IN FRESH ANIMAL TISSUES*

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Very limited information is available at the present time on the quantitative amounts of the essential aliphatic amino acids in various dietary constituents. Since the reports of Rose *et al.* (1, 2) on the qualitative requirements for eight amino acids, there has been much interest in the reinvestigation of the amino acid composition of proteins. Chemical methods have not usually been satisfactory for determining the amino acid content of foods directly, since the fat and carbohydrate present interfere with the chemical methods employed.

The use of *Lactobacillus arabinosus* in our laboratory as a test organism for vitamin analyses prompted us to investigate the adaptability of this organism to the amino acid analysis of foods. Methods of hydrolysis of animal tissues have been studied and it has been found that the leucine and valine content of the hydrolysates can be determined directly.

EXPERIMENTAL

The composition of the basal medium and the amounts of each constituent per tube are shown in Table I. Glucose, sodium acetate, and the amino acids are weighed out separately for each series of determinations. The other ingredients of the medium are kept as stock solutions preserved under toluene, in a refrigerator, when not in use. The following solutions are prepared as suggested by Snell and Wright (3): adenine, guanine, and uracil solution, riboflavin solution, and inorganic salts, Solutions A and B. The biotin (free acid) solution is adjusted with distilled water to a concentration of 0.1 γ per cc. The thiamine, calcium pantothenate, and pyridoxine solution is prepared by dissolving the vitamins in distilled water and diluting to a concentration of 100 γ of each per cc. of solution. Nicotinic acid and *p*-aminobenzoic acid solutions are prepared in a similar manner. Stock solutions of leucine and valine standards are prepared separately at a concentration of 1 mg. per cc. in distilled water.

The procedure for preparing the basal medium for leucine or valine assay

is as follows: Approximately 100 cc. of distilled water are added to the correct amounts of the amino acids, the mixture is warmed, and a small amount of 2 N hydrochloric acid is added to effect solution. After this solution is cooled, appropriate amounts of each of the other constituents are added. After neutralization (bromothymol blue indicator) the entire medium is adjusted to volume, for example to 500 cc. for 100 tubes, so that 5 cc. of medium are added to each assay tube. For the construction of the standard curve 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 cc. of amino acid standard solution are used per tube. Triplicate determinations are

TABLE I

Basal Medium for Valine and Leucine Assay with Lactobacillus arabinosus

Constituent	Amount per tube	Constituent	Amount per tube
	gm		*mg*
Glucose	0.2	*l*(+)-Glutamic acid	4
Sodium acetate	0.2	*l*-Asparagine	4
	γ	*l*(+)-Lysine monohydro-	
Thiamine chloride	2	chloride	2
Ca pantothenate	2	*dl*-Threonine	2
Pyridoxine	2	*dl*-Valine*	2
Riboflavin	4	*dl*-Isoleucine	2
Nicotinic acid	2	*dl*-α-Alanine	2
p-Aminobenzoic acid	1	*l*(−)-Cystine	1
Biotin .	0 002	*l*(−)-Leucine*	1
Adenine, guanine, uracil	See text	*dl*-Methionine	1
Inorganic salts	" "	*dl*-Phenylalanine	1
		l(+)-Arginine monohydro-	
		chloride	0 5
		l(+)-Histidine monohydro-	
		chloride	0.5
		l(−)-Tyrosine	0.4
		l(−)-Tryptophane	0.4

The amino acids were purchased from Merck and Company, Inc
* Appropriate amino acid omitted from the basal medium

made at the six lower levels and duplicate determinations at the three higher levels of amino acid. Assay tubes for these standards are prepared each time from the stock solutions described above, these being prepared at a concentration of 25 γ per cc. for *l*-leucine and 50 γ per cc. for *dl*-valine.

Duplicate tubes at three levels of hydrolysate concentration are used routinely. The volume of medium in each tube is adjusted to 10 cc. by the addition of distilled water. The tubes are plugged with cotton, autoclaved for 10 to 15 minutes at 15 pounds pressure, and cooled.

To prepare the inoculum, a transfer of *Lactobacillus arabinosus* is made from the stock culture (grown on yeast-dextrose-agar) to a tube of previously prepared medium. After incubation for 18 to 36 hours at 37°, the cells are centrifuged down and the liquid is decanted. The cells are suspended in approximately 10 cc. of isotonic saline solution, and the assay tubes are inoculated aseptically with a drop of the saline suspension. The tubes are incubated at 37° for 72 hours and the growth response is measured by titrating the entire contents of each tube with 0.1 N sodium hydroxide, bromothymol blue being employed as the indicator. Typical standard curves for leucine and valine are shown in Figs. 1 and 2.

The animal tissues used for amino acid analysis were prepared by thorough grinding and mixing to assure homogeneity. The samples were
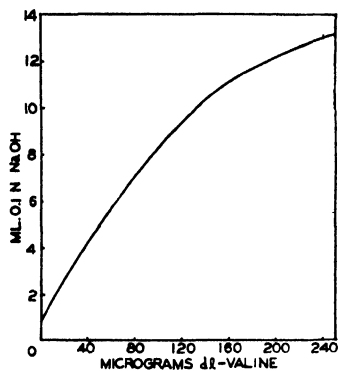
FIG. 1.

FIG. 2.

FIG. 1. Typical standard curve obtained for *dl*-valine.
FIG. 2. Typical standard curve obtained for *l*-leucine.
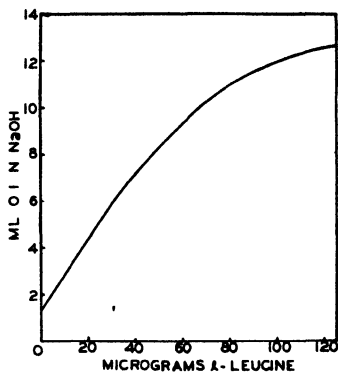
then stored in dark colored bottles in a cold room at −4°. The percentage of nitrogen in the samples was determined in duplicate by the Kjeldahl method and the protein content of the tissues was calculated by multiplying by 6.25.

A detailed study of hydrolysis methods was carried out to establish a suitable procedure to give maximum liberation of the amino acids. The effect of acid concentration and length of time of hydrolysis was investigated. In each case a 1 gm. sample of fresh tissue was hydrolyzed with 25 cc. of reagent. After hydrolysis the samples were adjusted to a known volume, filtered, and a suitable aliquot was neutralized and diluted to 100 cc. preparatory to amino acid analysis.

Treatment with hydrochloric acid resulted in more rapid hydrolysis than with sulfuric acid at the same normality, a result which is in agreement

with the work of Vickery (4) on purified proteins. The effect of time of hydrolysis on the amounts of leucine and valine liberated is shown in Fig. 3. The following hydrolysis procedures were found to give satisfactory results: autoclaving at 15 pounds pressure per sq. in. for 5 to 10 hours with 2 N hydrochloric acid, refluxing for 24 hours with 2 or 4 N hydrochloric
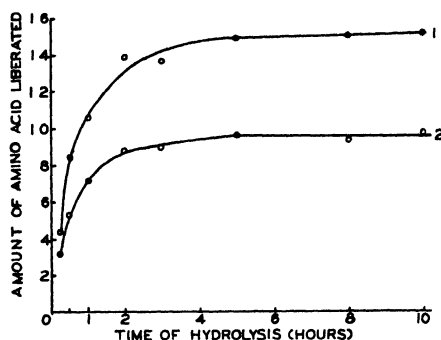
Fig. 3. Effect of time of hydrolysis of a sample of fresh veal tissue autoclaved with 2 N HCl at 15 pounds pressure per sq in Curve 1, the amount of leucine liberated, expressed as the per cent of leucine in the fresh tissue, Curve 2, the amount of valine liberated, expressed as the per cent of valine in the fresh tissue.

TABLE II

Leucine and Valine Content of Fresh Muscle and Organ Tissue and Muscle and Organ Tissue Protein

All values are given in per cent.

Tissue	No of samples	Protein	Valine in fresh tissue	Valine in protein	Leucine in fresh tissue	Leucine in protein
Pork	4	15 1	0 79	5 3	1.09	7 3
Veal.	5	21.0	1 11	5.3	1.54	7.3
Lamb	6	17 7	0.85	4 8	1 44	8.1
Beef	2	19.1	0 96	5 0	1.47	7.7
" liver	2	19 9	1 23	6 2	1.67	8 4
" kidney	2	14 6	0 78	5.3	1 15	7.9
" heart	1	16 1	1.01	6 3	1 35	8.4
" brain.	2	10.7	0 51	4 8	0.79	7.4
" tongue	1	16 0	0 79	4.9	1.23	7.7

acid, and refluxing for 24 hours with 5 N sulfuric acid. Autoclaving for 5 hours with 2 N hydrochloric acid was used for all subsequent assays. Shorter periods of refluxing were not investigated. Recoveries of added leucine and valine were employed to check the stability of these amino acids during the hydrolysis procedures.

The percentage of *l*-leucine and *l*-valine in several samples of pork, veal, lamb, and beef tissues has been determined. The results in each case are calculated on the basis of 100 per cent activity for the *l* isomer and 0 per cent for the *d* isomer; *i.e.*, 50 per cent for the *dl* mixture. These results are shown in Table II.

<center>DISCUSSION</center>

The constituents of the basal medium, except for the amino acids, are similar to those used by Snell and Wright (3) save for an increase in the glucose and sodium acetate content (Krehl *et al.* (5)). Adenine, guanine, uracil, and the inorganic salts are added in the concentrations suggested by Snell and Wright, but the vitamin levels have been increased in some cases. The amino acid mixture is similar to that reported by Shankman (6). No attempt was made to establish the exact levels of all of the amino acids which are necessary to support maximum growth. It will be noted that slight differences occur in the amino acid composition of the basal media used by various workers. Variations in the purity of the amino acids used, or differences in strains of *Lactobacillus arabinosus*, may account for differences in the amino acid requirements reported for this organism.

The maximum growth obtained with *Lactobacillus arabinosus* on the synthetic medium used in this work is similar to that obtained on a casein hydrolysate when tryptophane and cystine are added. The curves obtained for leucine and valine are similar to those reported by Kuiken *et al.* (7). Similar microbiological procedures have been reported by Shankman (6), Hegsted (8), and McMahan and Snell (9). Serine and proline were added to the basal medium and no increase in titrations was observed. These amino acids were accordingly omitted from the basal medium.

Excellent checks were obtained at different levels of sample regardless of the portion of the standard curve represented by the titrations obtained. A few representative assays are shown in Table III. Duplicate analyses usually checked within 5 per cent and all values were checked within 10 per cent. Recoveries of valine and leucine were carried out with the earlier experimental work on hydrolysis and occasionally with later assays. Twenty determinations of the recovery of valine averaged 101 per cent; range, 87 to 112 per cent and twelve for the recovery of leucine averaged 103 per cent; range 94 to 118 per cent.

In preliminary work a *dl*-leucine standard was used. Comparisons of different samples of *dl*-leucine showed that a variation in activity was obtained. A sample of *l*-leucine and three different *dl*-leucine standards were compared; the *dl* isomers were found to have from 33 to 41 per cent activity rather than the theoretical 50 per cent activity. Since three samples of *dl*-leucine exhibited less than 50 per cent activity as compared

to the *l* isomer, each sample was tested for moisture by drying it over phosphorus pentoxide for 6 hours at 100°, and 0.1 mm. pressure. No detectable loss in weight occurred. The *l* isomer and a new *dl*-leucine sample gave identical standard curves; therefore, no further attempt was made to find out why the older *dl* isomers were less than 50 per cent as active as the *l* isomer. It is very likely that they were contaminated with other amino acids, as has been observed by Hegsted and Wardwell (10).

A *dl*-valine standard was used throughout the experimental work and three *dl*-valine standards were found to possess the same activity for

TABLE III

Detailed Analysis of Animal Tissues

Sample	*l*-Valine				*l*-Leucine			
	Hydroly-sate per tube	Titration (0 1 N NaOH)	Calculated *l*-valine per tube	Valine in sample	Hydroly-sate per tube	Titration (0 1 N NaOH)	Calculated *l*-leucine per tube	Leucine in sample
	ml	*ml*	γ	*per cent*	*ml.*	*ml*	γ	*per cent*
Beef round	1	3.4	14.5	0.97	1	4.5	19.0	1.27
	1	3.3	14.0	0.94	1	4.7	21.0	1.40
	2	5 6	29.0	0.97	2	7.4	43.0	1.43
	2	5.5	28.0	0 94	2	7.5	44.0	1.46
	3	7.4	43.0	0.95	3	9.6	59.0	1.31
	3	7.5	44.0	0.98	3	9.4	57.0	1.26
Average				0.96				1.36
Beef liver	1	3.7	16.0	1.06	1	5.1	23.0	1.53
	1	3.8	17.0	1.13	1	5.3	25.0	1.66
	2	6.0	32.0	1.06	2	8.5	53.0	1.76
	2	6.2	33.0	1.10	2	8.5	53.0	1.76
	3	8.1	49.0	1.09	3	11.1	85.0	1.88
	3	7.9	48.0	1.06	3	10.6	78.0	1.73
Average..				1.08				1.72

Lactobacillus arabinosus. A sample of *l*-valine[1] and the *dl* standard were compared and the *dl* isomer was found to have 50 per cent activity; therefore, all values for *l*-valine were calculated on the basis of 50 per cent activity for the *dl* mixture. These observations suggest that *dl* isomers, when used as standards, should be checked against the pure *l* isomer whenever possible in experimental work of this type.

The results in Fig. 3 show that very rapid increases in the amounts of leucine and valine liberated occur during the first 2 hours of hydrolysis under the conditions used. Slight additional increases were observed in

[1] Kindly supplied by Merck and Company, Inc.

the period from 2 hours to 5 hours, but no further change occurred in 10 hours of autoclaving. Similar hydrolysis studies have been reported with purified protein hydrolysates by Hess and Sullivan (11), Vickery (4), and McMahan and Snell (9). Kuiken *et al.* (7) and Hegsted (8) refluxed protein samples with 5 N sulfuric acid and 6 N hydrochloric acid, respectively, for 24 hours. No mention was made as to criteria for completeness of hydrolysis, but these procedures should give satisfactory results.

The adaptability of microbiological methods to the determination of amino acids in fresh tissues greatly simplifies amino acid determinations. Furthermore, the results are obtained on the foods directly, and the amounts of leucine and valine in the crude material need not be calculated from amino acid values obtained on isolated proteins as has customarily been done when chemical methods of analysis are employed. The possibility that some amino acids are lost when the water-soluble nitrogenous constituents are discarded is also eliminated. The water-soluble fraction obtained in the preparation of meat protein has been estimated by Beach *et al.* (12) to contain from 8 to 14 per cent of the total nitrogen.

Preliminary results indicate that satisfactory results are obtained when leucine and valine are determined directly on cereal and legume hydrolysates. Hydrolysis of the crude material may not be applicable to the satisfactory determination of some of the other amino acids. The necessity of studies of the conditions of hydrolysis used in developing adequate procedures for the determination of amino acids in crude materials cannot be overemphasized.

The values obtained for valine are somewhat higher than those reported by Block and Bolling (13), who employed chemical methods of determination. They report 0.7 per cent valine in fresh meat (calculated from protein analysis) and 3.4 per cent valine in the muscle protein, whereas we found an average of 0.93 per cent valine in fresh beef, veal, lamb, and pork muscle and 5.1 per cent in the muscle protein. The amount of leucine reported by Block and Bolling was 2.4 per cent in fresh meat and 12.1 per cent in the muscle protein. In this case our results were lower, since an average of 1.4 per cent leucine was found in the muscle tissue and 7.6 per cent in the muscle protein. It can readily be seen (Table II) that the percentage of valine and leucine in the fresh tissues varies with the protein content, but the amino acid composition of the protein is relatively constant. This has also been shown to be true for several other amino acids by Beach *et al.* (12).

Purified casein was also investigated and Table IV presents a comparison of the results together with values from the literature. Since more extensive information is available for casein than for most other proteins, a critical comparison can be made of the results obtained by several workers.

It is recognized that the analyses were made on different samples of casein; however, the wide variations emphasize the importance of proper standardization of hydrolysis procedures and methods of amino acid analysis.

TABLE IV

Leucine and Valine Content of Casein

Amino acid	Method of analysis	Results	Reference
		per cent	
Leucine	Chemical	9.7	Foreman (14)
"	"	9.35	Van Slyke (15)
"	"	10.5	Abderhalden (16)
"	"	14.8	Block and Bolling (17)
"	Microbiological	9.6	Present work
"	"	9.27	Kuiken et al. (7)
"	"	7.36	Hegsted (8)
Valine	Chemical	7.93	Foreman (14)
"	"	5.2	Block and Bolling (17)
"	Microbiological	6.2	Present work
"	"	6.25	Kuiken et al. (7)
"	"	6.6	McMahan and Snell (9)
"	"	5.0	Hegsted (8)

SUMMARY

Satisfactory standard curves are obtained for leucine and valine on a synthetic amino acid medium when *Lactobacillus arabinosus* is used as the test organism.

Results of hydrolysis studies show that fresh muscle tissues can be hydrolyzed directly for leucine and valine analysis without preliminary removal of fat, moisture, and water-soluble constituents.

Satisfactory hydrolysis of animal tissues was obtained by autoclaving with 2 N HCl for 5 to 10 hours.

Fresh muscle tissues contain an average of 0.93 per cent valine and 1.4 per cent leucine. Muscle and organ tissue proteins contain an average of 5.3 per cent valine and 7.7 per cent leucine.

BIBLIOGRAPHY

1. Rose, W C , Haines, W J , and Johnson, J. E , *J Biol Chem* , **146, 683** (1942).
2. Rose, W. C , Haines, W. J , Johnson, J. E , and Warner, D. T , *J Biol. Chem.*, **148,** 457 (1943).
3. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139,** 675 (1941).
4. Vickery, H. B , *J. Biol. Chem.*, **53,** 495 (1922).
5. Krehl, W. A., Strong, F. M , and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **11,** 316 (1943).
6. Shankman, S., *J. Biol. Chem.*, **150,** 305 (1943).

7. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
8. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
9. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
10. Hegsted, D. M., and Wardwell, E. D., *J. Biol. Chem.*, **153**, 167 (1944).
11. Hess, W. C., and Sullivan, M X., *Arch. Biochem.*, **3**, 53 (1943).
12. Beach, E. F., Munks, B., and Robinson, A , *J. Biol. Chem.*, **148**, 431 (1943).
13. Block, R. J., and Bolling, D., *Arch. Biochem* , **3**, 217 (1943).
14. Foreman, F. W., *Biochem. J* , **13**, 378 (1919).
15. Van Slyke, D. D., *Ber. chem. Ges.*, **43**, 3170 (1910)
16. Abderhalden, E , *Z. physiol. Chem* , **44**, 23 (1905)
17. Block, R. J , and Bolling, D., The determination of the amino acids, Minneapolis, revised edition (1940).

MECHANISM OF PRODUCTION OF VITAMIN K DEFICIENCY IN RATS BY SULFONAMIDES

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The use of sulfonamides in purified diets of rats has resulted in the production of vitamin deficiencies. The first identified deficiency thus induced was that of vitamin K produced by sulfaguanidine and succinyl sulfathiazole and was reported by Black *et al.* (1). It was plausible to postulate that this deficiency was a result of the inhibition·by the sulfonamides of bacterial synthesis of vitamin K in the intestinal tract. It is known that coliform organisms produce vitamin K *in vitro* (2) and that the feces of rats on a vitamin K-free ration contain vitamin K (3). Sulfaguanidine and succinyl sulfathiazole reduce the coliform count in the feces of rats (4). *p*-Aminobenzoic acid, which antagonizes sulfonamide bacteriostasis, was also shown to prevent the production of vitamin K deficiency by sulfaguanidine (1).

Recently other data (5, 6) have been reported which support this hypothesis of sulfonamide inhibition of intestinal bacterial synthesis of vitamin K. We (5) have shown that sulfapyrazine, sulfadiazine, and sulfathiazole are more effective than sulfaguanidine, succinyl sulfathiazole, and sulfanilamide in producing vitamin K deficiency and that this order of effectiveness of these sulfonamides approximates the order of their bacteriostatic potency against intestinal coliform organisms as reported by White (7). In addition it was observed (5) that factors such as absorption, utilization, and alteration of requirements of vitamin K did not appear to be significant elements in the production of vitamin K deficiency by these sulfonamides. Day *et al.* (6) found that cecectomy increased the incidence of vitamin K deficiency in rats fed diets containing succinyl sulfathiazole.

Certain evidence has been presented by Black *et al.* (1) which they interpret to be inconsistent with the hypothesis limiting the sulfonamide action to the intestinal tract. These workers found that *p*-aminobenzoic acid even when administered parenterally antagonized the vitamin K deficiency produced by sulfaguanidine. They reasoned, therefore, that this sulfaguanidine action "cannot be explained on the basis of changes in intestinal flora alone, but may be due to a toxic action . . . on certain tissues of the rat, which is counteracted by *p*-aminobenzoic acid." They concluded that the exact mode of action of sulfaguanidine is, therefore, obscure.

The present study was undertaken to gather more evidence concerning the mechanism of production of vitamin K deficiency by sulfonamides. We have confirmed the findings of Black *et al.* (1) that parenterally administered *p*-aminobenzoic acid prevents the development of vitamin K deficiency but in addition we have found that *p*-aminobenzoic acid so administered appears in high concentrations in cecal contents. Thus the findings of Black *et al.* do not necessarily conflict with the hypothesis of sulfonamide inhibition of intestinal bacterial synthesis of vitamin K. In addition new and more direct evidence is presented here in support of this hypothesis.

Methods

The techniques and diets used here were generally the same as those previously described (5). Weanling, albino rats were fed a purified control or experimental diet. The diets were identical except that, in the experimental, 1 per cent of the dextrose was replaced by an equal weight of the sulfonamide drug.

Prothrombin time was determined by a micromethod on whole blood from the tail. The *"prothrombin level"* was derived by dividing the average prothrombin time of a group of control rats by the prothrombin time of the experimental rat; the result was multiplied by 100 and expressed as a per cent. The term *hypoprothrombinemia* was applied only to those rats whose "prothrombin levels" had fallen below 30 per cent.

Determinations of sulfadiazine were by the method of Bratton and Marshall (8) with a photoelectric colorimeter. Blood determinations were made on blood obtained by decapitation or by a micromethod (9) on 0.02 cc. of tail blood. Cecal contents were homogenized with 200 cc. of water in a Waring blendor for 5 minutes and then made up with vigorous shaking to a volume of 500 cc. containing 100 cc. of 15 per cent trichloroacetic acid. Determinations were made on the clear filtrates and concentrations were expressed on a wet weight basis. The water fraction of cecal contents was generally from 75 to 85 per cent.

p-Aminobenzoic acid was assayed by the microbiological method of Landy and Dicken (10) on cecal contents dried for 10 hours at 80° and powdered.[1] The test organism was *Acetobacter suboxydans*. Only free *p*-aminobenzoic acid was determined. The dried cecal contents were extracted with hot water in preparation for the assay. The microbiological method was preferred to diazo chemical methods because the latter were not sufficiently sensitive for the entire range of values.

The technique for the assay of vitamin K activity of crude material has

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[1] Lieutenant M. Landy, Army Medical School, performed the *p*-aminobenzoic acid assays.

been described previously (5). The responses of hypoprothrombinemic rats to a sample of feces or cecal contents were compared with responses of similar rats to doses of pure vitamin K. "Increases" over the pretreatment "prothrombin levels" of 60 per cent or more, of 20 to 60 per cent, and of less than 20 per cent were considered to represent the following respective degrees of vitamin K (2-methyl-1,4-naphthohydroquinone diacetate) activity: 5 γ or greater, 2 to 4 γ, and less than 2 γ. Aliquots of a suspension of feces or cecal contents gave similar values in different rats. Feces were collected and frozen daily. Cecal contents were removed from a rat sacrificed by decapitation and used immediately for assay purposes; in a few instances they were frozen and used later. The materials were suspended in water and administered by stomach tube. Prothrombin determinations were made immediately before and 24 hours after administration of the assayed material.

Sodium sulfadiazine in an aqueous solution containing 10 gm. per 100 cc. was administered subcutaneously each day at a dose level of 0.5 mg. per gm. of body weight. p-Aminobenzoic acid in a neutralized solution containing 0.1 or 1.0 gm. per 100 cc. was administered subcutaneously each day at levels of 5 or 50 γ per gm. of body weight.

Results

Hypoprothrombinemia and hemorrhage were produced by the daily subcutaneous administration of sodium sulfadiazine and this effect was prevented by p-aminobenzoic acid also administered subcutaneously (Table I). p-Aminobenzoic acid at a dose level of 50 γ per gm. of body weight almost completely antagonized the development of the vitamin K deficiency.

The production and prevention of vitamin K deficiency by parenterally administered agents does not exclude the intestinal tract as a locus of these actions. This is shown by a study of the concentrations of sulfadiazine and p-aminobenzoic acid in the cecal contents of rats injected subcutaneously with these agents.

Sulfadiazine determinations (Table II) were made of the cecal contents of rats injected daily with sulfadiazine (0.5 mg. per gm. of body weight) and of rats ingesting diets containing from 0.25 to 1.0 per cent sulfadiazine. It was found that sulfadiazine concentrations in the cecal contents of the injected rats were of the same general order as those found in rats ingesting a diet containing 0.5 per cent sulfadiazine and the incidence of vitamin K deficiency was similar in both groups. In rats with low concentrations of sulfadiazine in the cecum, vitamin K deficiency did not appear, but in rats with high concentrations of sulfadiazine in the cecum, vitamin K deficiency was noted in most cases. Thus in a group of seven rats with cecal sulfadiazine concentrations under 110 mg. per 100 gm., none developed hypo-

TABLE I

Production of Vitamin K Deficiency by Sodium Sulfadiazine Administered Subcutaneously and Prevention by p-Aminobenzoic Acid Administered Subcutaneously

Type of diet	No. of rats	Lowest "prothrombin levels"* reached by individual rats	Average of lowest "prothrombin levels"
		per cent	*per cent*
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight	10	4, 4, 4, 16, 25, 38, 39, 53, 67, 97	35
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight + 5.0 γ p-amino-benzoic acid per gm. body weight	5	20, 83, 85, 88, 96	75
Control diet + sodium sulfadiazine, 0.5 mg. per gm. body weight + 50.0 γ p-amino-benzoic acid per gm. body weight	5	81, 83, 88, 96, 97	89
Experimental diet containing 1% sulfadiazine	5	8, 11, 12, 14, 19	13
Experimental " " 1% sodium sulfadiazine	5	4, 4, 10, 13, 26	11

* Determinations were made at 1, 2, and 3 weeks.   Litter mates were used in this experiment.

TABLE II

Concentrations of Sulfadiazine in Cecal Contents and Blood

Type of diet	No of rats	Sulfadiazine (free)* concentrations in cecal contents (wet weight basis)		Sulfadiazine (free)* concentrations in blood		No. of rats with hypoprothrombinemia ("prothrombin level" = <30 per cent)
		Average	Range	Average	Range	
		mg. per 100 gm.	*mg. per 100 gm.*	*mg. per cent*	*mg. per cent*	
0.25% sulfadiazine	5	76	64– 103	16	16–16	0
0.50% "	4	138	43– 245	32	27–34	1
0.75% "	3	704	522– 868	43	41–44	2
1.0% " ..	10	1609	1160–1850	48	34–54	10
Control diet + sodium sulfadiazine subcutaneously (daily), 0.5 mg. per gm. body weight.. .	5	126	104– 155	35	30–46	2

* Determinations were made from 2 to 4 weeks after the start of the experiment.

prothrombinemia. Of twenty-five rats, with sulfadiazine concentrations over 110 mg. per 100 gm. twenty developed hypoprothrombinemia. The other five rats had the following concentrations of sulfadiazine in their cecal contents: 118, 129, 147, 245, and 723 mg. per 100 gm.

The concentration of *p*-aminobenzoic acid (subcutaneously administered) in the cecal contents was determined in rats on a control diet

TABLE III

Estimation of Free p-Aminobenzoic Acid in Cecal Contents

The values are given in micrograms per gm. (dry weight).*

Control rats†	Rats injected daily for 10 days with *p*-aminobenzoic acid, 5 γ per gm. body weight	Rats injected daily for 10 days with *p*-aminobenzoic acid, 50 γ per gm. body weight
0.144	7.1	31.2
0.408	6.8	20.2
0.216	7.3	17.0
0.168	7.5	19.8

* Stool moisture content varied from 77 to 84 per cent.

† Four groups of three litter mates were used in this experiment.   Lieutenant M. Landy, Army Medical School, performed the *p*-aminobenzoic acid assays.

TABLE IV

Vitamin K Activity of Feces and Cecal Contents

Type of diet	Material assayed	Amount of material assayed	No. of rats	"Prothrombin levels" of assay rats before and after administration of test material*		Approximate vitamin K† activity of material assayed
Control	Feces	1/10 of 5 day sample	3	Before	8, 12, 12	>50 γ (for total 5 day sample)
				After	94, 100, 86	
		1/20 of 5 day sample	2	Before	11, 25	
				After	78, 108	
Experimental‡	"	Total 5 day sample	8	Before	19, 19, 20, 5, 6, 6, 7, 21	<2 γ (for total 5 day sample)
				After	37, 26, 53, 15, 6, 14, 31, 11	
Control	Cecal contents	¼ of total	4	Before	15, 19, 27, 27	>12 γ (for total cecal contents)
				After	51, 51, 78, 84	
Experimental‡	Cecal contents	Total	5	Before	25, 20, 23, 10, 17	<2 γ (for total cecal contents)
				After	27, 15, 27, 12, 8	

* Values from the same individual rats are tabulated in the same order on the "Before" and "After" lines.

† 2-Methyl-1,4-naphthohydroquinone diacetate.

‡ The experimental diet contained either 1 per cent sulfapyrazine or 1 per cent sulfadiazine.

(Table III).   A daily parenteral dose of 5 γ per gm. of body weight resulted in a 30-fold increase in the concentration of *p*-aminobenzoic acid in the

cecal contents, while a dose of 50 γ per gm. of body weight increased the p-aminobenzoic acid concentration in the cecum about 100-fold.

Vitamin K activity of feces and cecal contents was assayed on hypoprothrombinemic rats. The responses of these rats were compared with those of rats given known amounts of pure vitamin K. A large difference was found between the vitamin K activity of feces of rats on sulfonamide diets and those of rats, usually litter mates, on the control diets (Table IV). The total feces for 5 days obtained from each of eight rats on experimental diets gave responses of less than 2 γ of vitamin K activity in seven cases and a response of 2 to 4 γ in one case. Similar values were obtained from two rats with hypoprothrombinemia due to subcutaneously injected sulfadiazine. When the total feces for 5 days of these two rats were fed to hypoprothrombinemic rats with "prothrombin levels" of 13 and 11 per cent, responses to levels of 56 and 17 per cent respectively were obtained. This indicates 2 to 4 γ of vitamin K activity in one rat and less than 2 γ in the other. 5 day fecal samples from control rats of similar age and weight were found to contain more than 50 γ of vitamin K activity. Wet and dry weights of fecal samples from experimental and control rats were of the same order of magnitude.

Since vitamin K synthesis may occur in feces at room temperature during intervals between daily collections and refrigeration, vitamin K assays were made of cecal contents. Comparable rats on experimental and control diets were sacrificed. Cecal contents of four control rats each showed about 12 γ of vitamin K activity, those of five rats on a deficiency-producing, sulfonamide diet each possessed no demonstrable vitamin K activity or less than 2 γ. One rat injected subcutaneously with sulfadiazine also had less than 2 γ of vitamin K activity in its cecal contents.

<center>DISCUSSION</center>

The vitamin K activity of cecal contents (or collected feces) was very low in rats with vitamin K deficiency produced by the oral or parenteral administration of sulfonamides. The results obtained show that the cecal contents of such vitamin K-deficient rats possess no demonstrable vitamin K activity or less than 2 γ, while the cecal contents of control rats possess 12 γ or more of vitamin K activity. This finding points to the inhibition of intestinal bacterial synthesis of vitamin K as an important factor in the production of vitamin K deficiency by sulfonamides.

This hypothesis for the mechanism of production of vitamin K deficiency by sulfonamides is further supported by other data presented here. The production of vitamin K deficiency by orally or parenterally administered sulfadiazine was found to be definitely related to the sulfadiazine concentration in the cecal contents. It was also observed that p-aminobenzoic

acid injected subcutaneously appeared in significant amounts in the cecum and prevented the production of a vitamin K deficiency by subcutaneously administered sulfadiazine. The known antagonism of sulfadiazine bacteriostasis by p-aminobenzoic acid provides a plausible basis for the action of p-aminobenzoic acid in preventing vitamin K deficiency.

We have reported previously on the parallelism between the order of bacterial potency of a series of sulfonamides and the order of their effectiveness in production of a vitamin K deficiency (5). It was also reported by us that factors of absorption, utilization, and altered requirements of vitamin K did not appear to be significant elements in the production of a vitamin K deficiency by sulfonamides. Day *et al.* (6) noted that cecectomy facilitated the development of a vitamin K deficiency in rats fed succinyl sulfathiazole diets. These findings are all in keeping with those presented at this time.

It should be pointed out that the mechanism of production of other vitamin deficiencies by sulfonamides may not be the same as that considered here for the production of a deficiency of vitamin K.

SUMMARY

1. The cecal contents and collected feces of rats with vitamin K deficiency produced by sulfonamides showed either very slight or no vitamin K activity. The cecal contents and collected feces of control rats possessed much greater vitamin K activity.

2. Sulfadiazine administered subcutaneously resulted in a vitamin K deficiency. The production of the deficiency by orally or parenterally administered sulfadiazine was closely related to the concentration of sulfadiazine in the cecal contents.

3. p-Aminobenzoic acid administered subcutaneously antagonized the vitamin K deficiency produced by subcutaneously administered sulfadiazine. p-Aminobenzoic acid injected into rats on a control diet appeared in the cecum in significant concentrations.

4. These findings together with other available data indicate that the inhibition of intestinal bacterial synthesis of vitamin K is the dominant factor in the production of vitamin K deficiency in rats by the sulfonamides used in these studies.

BIBLIOGRAPHY

1. Black, S., Overman, R S , Elvehjem, C A , and Link, K. P., *J. Biol. Chem.*, **145**, 137 (1942).
2. Orla-Jensen, S., Orla-Jensen, G. D , Dam, H., and Glavind, J., *Zentr. Bakt.*, *2. Abt* , **104**, 202 (1941); *Chem. Zentr.*, **1**, 1155 (1942)
3. Greaves, J. D., *Am. J. Physiol.*, **125**, 429 (1939).

4. Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **52**, 276 (1943).

5. Kornberg, A., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, *U. S. P. H. S.*, **59**, 832 (1944).

6. Day, H. G., Wakim, K. G., Krider, M. M., and O'Banion, E. E., *J. Nutr.* **26**, 585 (1943).

7. White, H. J., *Bull. Johns Hopkins Hosp.*, **71**, 213 (1942).

8. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).

9. Marshall, E. K., Jr., and Cutting, W. C., *Bull. Johns Hopkins Hosp.*, **63**, 328 (1938).

10. Landy, M., and Dicken, D. M., *J. Biol. Chem.*, **146**, 109 (1942).

RESPIRATION IN THE ORANGE

A STUDY OF SYSTEMS RESPONSIBLE FOR OXYGEN UPTAKE

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In a study of respiration in the orange (*Citrus sinensis*) an attempt has been made to determine the type of oxygen-absorbing system present in the various tissues of the orange fruit, with particular emphasis on the flavedo. Oranges were classified as a peroxidase fruit by Onslow (1). Recently Davis (2) prepared peroxidase extracts from the different tissues of the navel orange and reported highest values in the flavedo. Qualitative tests with guaiacol and benzidine have confirmed this observation. In the present study, measurements of oxygen uptake by the Warburg technique show that a system resembling cytochrome·oxidase is involved. Dehydrogenase-like activity, as measured by methylene blue reduction, has also been studied.

Methods

Respiration measurements were made with the customary Warburg manometer and conical reaction flasks. The Q_{O_2} or c.mm. of O_2 consumed was calculated on the basis of 1 mg. of dry weight per hour. The oranges were supplied by the Citrus Experiment Station at Riverside. Weekly samples were received from the same tree and stored at 0°. The fruit was washed with cold water, adhering water removed, and the tissue desired removed with a sharp blade. Flavedo samples were taken from the middle, to avoid tissue with a greenish tint. Cross-sections with a thickness of approximately 0.7 mm. were made by free-hand slicing. The sections were rinsed with distilled water[1] before transference to the Warburg vessels which contained 2 ml. of 0.2 M phosphate buffer. The samples weighed 200 mg. when fresh, containing about 50 mg. of dry weight.

Dehydrogenase activity was studied by methylene blue reduction in Thunberg tubes. The juice was prepared by mincing the sample, freezing and thawing, grinding with sand in a mortar, and finally pressing in a hand press. The juice was mixed then with infusorial earth and filtered through asbestos. The clear juice was decolorized with norit A, and again filtered. The clarified juice was kept under toluene at 0°. The final volume in the tubes was 2 ml., consisting of 0.2 ml. of 0.02 per cent methylene blue,

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[1] The distilled water in these solutions was redistilled once in Pyrex.

0.5 ml. of the clarified tissue juice, and 1.3 ml. of 0.2 M potassium phosphate. The tubes were evacuated for 1 minute with a Nelson pump and allowed to stand for 20 minutes to reach room temperature before mixing. A tube containing all the reagents after decolorizing was used for comparison. As the reaction was found to be catalyzed by light, the reduction period was measured under artificial light with a 75 watt Mazda lamp.

<center>EXPERIMENTAL</center>

Determination of Respiratory Quotient—The respiratory quotient was determined in 0.2 M phosphate buffer of pH 5.0. In six determinations, the average was 1.33 at the start and 1.09 after 2 hours.
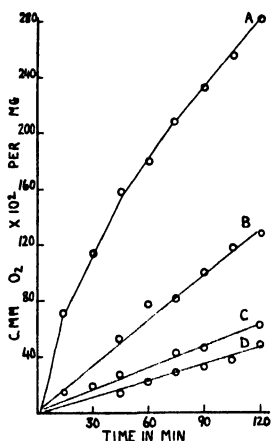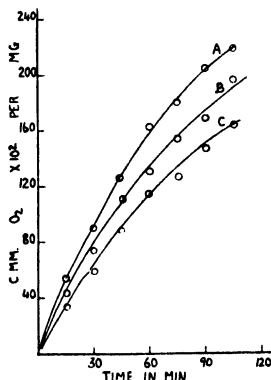
<center>FIG 1   FIG. 2</center>

FIG. 1. Comparative activity of different tissues. Curve A, flavedo tissue; Curve B, albedo tissue; Curve C, juice sacs; Curve D, carpellary membranes.

FIG. 2. Effect of size of flavedo section on oxygen uptake. Curve A, 3 mm.; Curve B, 6 mm ; Curve C, 12 mm. in diameter

General Characteristics of System. The Sample—Flavedo sections prepared in the same manner, but obtained from different locations on the orange, showed different oxygen uptake, with highest activity near the stem end, and lowest near the blossom end. Q_{O_2} values were 1.35, 1.17, and 0.92 respectively. Variability among oranges is of considerable magnitude, ranging from 1.60 to 2.10 for twelve navels and 1.10 to 1.55 for twelve Valencias taken at random. Maturity, elapsed time, and the uncontrollable variables are doubtless responsible for this. Comparative activities for the different tissues are shown in Fig. 1 for navels. In each, the flavedo has the highest and the carpellary membranes the lowest activity. An increase was noted in oxygen uptake with decreasing size,

but similar trends were obtained for the values taken at comparable time intervals (Fig. 2). Although decreasing the length of the cross-sections resulted in an increase in Q_{O_2}, the activity was lost if the tissue was finely ground with sand.

Washing—When flavedo sections are washed in running water, their activity decreases gradually and finally ceases completely after 24 hours. When the tissues were washed for 24 hours with mechanical stirring and with a stream of air passing through the liquid, between 30 and 40 per cent of the initial activity was retained.

To determine whether this difference in activity is associated with fermentation, flavedo sections were left in the Warburg vessels for 22 hours under an atmosphere of purified nitrogen. An average Q_{CO_2} of 1.66 for the first 3 hours was observed. The original Q_{O_2} was 1.33. After fermentation
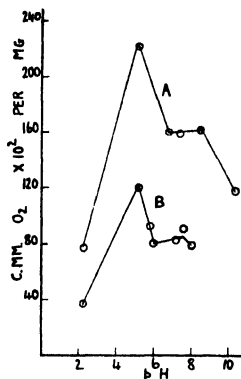
FIG. 3. Effect of pH on the oxygen uptake of flavedo. Curve A, 1st hour; Curve B 2nd hour.

was allowed to continue for 22 hours, the value for Q_{O_2} dropped to 0.16 c.mm. for the 1st hour and no further oxygen uptake was observed after 2 more hours.

Heat—When flavedo sections were heated for 2 minutes in boiling water or buffer, neither the tissue alone nor the fluid showed any oxygen uptake. Heating was also conducted in an atmosphere of purified nitrogen, but the power of oxygen uptake was lost.

Effect of pH—The pH of the expressed juice of both flavedo and albedo tissues ranges from 4.9 to 5.4. When the oxygen uptake of flavedo sections was determined at different pH values between pH 3.0 and 7.0, an optimum near pH 5.0 was observed for both varieties. Above pH 7, the oxygen uptake remains relatively constant, but rises slightly near pH 8 (Fig. 3).

Effect of Substrates. Catechol—To establish the presence or absence of

polyphenolase in the flavedo, the effect of catechol was determined at the natural pH, 5.1, and at pH 8.15. The side arm of the flasks contained 0.2 ml. of 1 per cent catechol in aqueous solution. The initial oxygen uptake of the tissue was determined for 30 minutes before the catechol was tipped into the flasks. This caused no increase in the oxygen uptake at either pH.

Ascorbic Acid—The oxygen uptake of flavedo tissue in the presence of 2 mg. of ascorbic acid per ml. was compared with aqueous solutions of ascorbic acid at the same concentration, at different pH values (Table I). To ascertain whether ascorbic acid is partially oxidized, an extract was prepared by the method of Krishnamurthy and Giri (3). 1 ml. of extract was added to 1 ml. of buffer of pH 6.75, containing 5 mg. of ascorbic acid. The pH was 6.3 after the material was mixed. A buffer at this pH with the same quantity of ascorbic acid was used for comparison. The control vessels consumed 192 c.mm. of oxygen in 45 minutes, while there was

TABLE I

Oxygen Uptake by Flavedo with Ascorbic Acid

pH	C.mm oxygen consumed per 100 mg dry weight		
	Ascorbic acid control	Flavedo control	Flavedo with ascorbic acid
8.05	242	116	177
6.50	177	140	159
5.00	126	149	142

none in the presence of the extract, indicating apparently complete protection of the ascorbic acid.

p-Phenylenediamine—Experiments were conducted in phosphate buffer, pH 6.5; the side arm contained 0.2 ml. of a solution of the reagent (1.92 mg.) in 0.1 M buffer in 50 per cent alcohol. A blank contained the same reagents without the tissue. Autoxidation was negligible, about 5 c.mm. per hour. At the end of the experiment, both tissue and fluid were blue, and a 3-fold increase in oxygen uptake was observed. After 1 hour, p-phenylenediamine was added to one flask which had received none at the beginning. An increase in the oxygen consumption was also observed (Fig. 4).

The effect of p-phenylenediamine on the Q_{O_2} of the heated tissue, which had been boiled for 2 minutes, and on ground flavedo tissue was then determined. Oxygen consumption by the boiled tissue was similar to that of the blank, while the ground tissue, which did not consume oxygen in other experiments, gave values comparable with those of intact tissue alone, and the fluid was blue. The results are shown in Table II.

Experiments were also conducted to test the effect of the dye in the presence of potassium cyanide. In the presence of cyanide (0.01 M) neither tissue nor fluid became blue, and inhibition was about 75 per cent (Table III). The sensitivity to cyanide did not change when the test was repeated over an 8 week period.

Cytochrome c—The addition of 0.4 ml. of reduced cytochrome c solution, prepared from ox heart (4), to flavedo sections at pH 8, 6.5, and 5.0 caused an increase in oxygen uptake in all cases (Fig. 5). Cytochrome c is not autoxidized at these pH values and no blank, the cytochrome alone, was needed.

TABLE II

Oxygen Uptake by Flavedo with p-Phenylenediamine

Tissue	C.mm. oxygen consumed per 100 mg. dry weight	
	Experiment 1	Experiment 2
Fresh, intact.......	128	98
" " with dye ..	378	248
" ground.. . .	0	0
" " with dye.. ..	123	97
Boiled, with dye..	0	0–5

TABLE III

Effect of Cyanide on Oxygen Uptake with p-Phenylenediamine

C.mm. oxygen consumed per 100 mg. dry weight		
Flavedo	Flavedo with dye	Flavedo with dye and cyanide
107	320	81
108	294	79

Hydroquinone—Hydroquinone was added at the beginning and also during the course of the experiment. The side arm contained 0.2 ml. of 0.1 M aqueous hydroquinone. In neither case was an increase in uptake or brown discoloration noted. In three determinations, with and without hydroquinone, the average values after 2 hours at pH 7.0 were 0.77 and 0.75 respectively, and 0.66 and 0.61, when added after 1 hour. The oxidation of hydroquinone is catalyzed by cytochrome oxidase, and is a function of both the oxidase and cytochrome c (5). Since the hydroquinone was not oxidized, one may infer the absence of cytochrome c.

Other Possible Substrates—The following substances were also tested: 0.1 M solutions of malic, citric, and succinic acids, orange oil, citral and

limonene, citrin, riboflavin (with and without tissue dialysis), and glucose, fructose, maltose, galactose, and sucrose. None of these showed any effect on oxygen uptake by the flavedo, with the possible exception of succinic acid, for which a slight increase, about 10 per cent, in the Q_{O_2} was observed.

Effect of Inhibitors. Potassium Cyanide—Cyanide has an inhibitory effect ranging from 80 per cent at 10^{-2} M to 8 per cent at 10^{-3} M at pH 5.0, indicating a residual respiration of about 20 per cent (Fig. 6). It was also
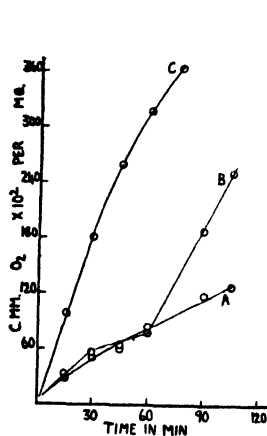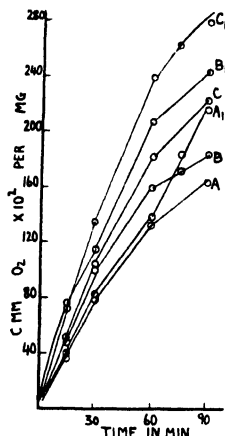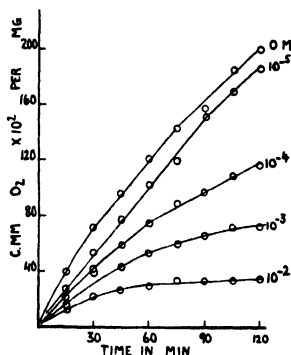
FIG. 4   FIG. 5   FIG. 6

FIG. 4. Effect of *p*-phenylenediamine on oxygen uptake in flavedo. Curve A, tissue alone; Curve B, with *p*-phenylenediamine added after 60 minutes, Curve C, with *p*-phenylenediamine added at the start.

FIG. 5. Effect of added cytochrome *c* on oxygen uptake. Curves A and A_1 at pH 8 0, without and with cytochrome; similarly Curves B and B_1 at pH 6 5; also Curves C and C_1 at pH 5 0.

FIG. 6. Effect of cyanide concentration on oxygen uptake of flavedo (pH 5.0).

found that the extent of inhibition depends on the pH, being about 80, 75, and 50 per cent at pH values of 5.0, 6.5, and 8.0 respectively.

Hydrogen Sulfide—To determine the effect of hydrogen sulfide, the tissues were allowed to respire for 1 hour in phosphate buffer at pH 5.1, containing 0.01 M sodium sulfide. About 0.2 ml. of the fluid was transferred to the side arm containing the alkali mixture ($KMnO_4$ and KI), and then $HgCl_2$ was mixed with the fluid. The flasks were shaken until constant readings of the manometer were obtained. This experiment was conducted only with Valencia oranges; the inhibition in two experiments was 75.5 and 80.3 per cent, respectively.

Sodium Azide—With 10^{-2} M azide, an inhibition of about 80 per cent was observed at pH 5.0, and 8 per cent at pH 8.0.

Carbon Monoxide—This experiment was conducted in light and in the dark, on tissue from the same orange, with a mixture of 80 per cent carbon monoxide and 20 per cent oxygen. In each experiment, the average of data from two flasks was used (Table IV). The inhibition is clearly reversible with light.

Spectroscopic Examination of Tissues—An attempt was made to observe the characteristic bands of cytochromes in the flavedo tissue. When a suspension of finely ground tissue in water was examined spectroscopically, no bands were observed. However, when sodium hydrosulfite was added to the suspension, a band at 563 mμ was easily recognized. When flavedo slices were examined, the band could be observed only after steeping the slices in hydrosulfite. Cytochrome b exhibits a band at 564 mμ, and the oxidase at 582 mμ (6). To determine whether the band was due to cyto-

TABLE IV

Effect of Carbon Monoxide on Oxygen Uptake by Flavedo

C mm oxygen consumed per 100 mg dry weight				Affinity ratio K*
In daylight		In dark		
In air	In gas mixture	In air	In gas mixture	
140	143	157	121	14.1
135	131	122	95	14.4

$$* K = \frac{\text{residual respiration}}{1 - \text{residual respiration}} \times \frac{CO}{O_2}.$$

chrome b, its position was checked against the known cytochrome b component in a yeast suspension. To the knowledge of the writer, none of the cytochromes has been observed before in a colored portion of the plant.

The characteristic bands of cytochrome c (550 and 520 mμ) were not observed. On the other hand when reduced cytochrome c was added to the flavedo suspension in the presence of $Na_2S_2O_4$, the band at 550 mμ was plainly visible, indicating that the failure to observe the band before was not due to a masking effect.

When cytochrome c was added in the absence of $Na_2S_2O_4$, the bands gradually disappeared, indicating a change from the reduced to the oxidized form, but when the tissue was heated before the cytochrome solution was added, the bands remained visible. This indicates that a heat-labile substance is responsible for the oxidation of added cytochrome c.

Success in viewing cytochrome bands depends upon two factors. As

the concentration of cytochromes is extremely low, a thick suspension is essential. This requires an intense beam to permit adequate transmitted light for visual inspection in the spectrometer. As light source, a Wotan lamp (6 volts, 5 amperes) was used, with a large condensing lens, and diaphragm, as used in a microscope assembly. Two instruments were tried, a small direct vision spectroscope, and a large Bausch and Lomb spectrometer. For best results on the bands *in situ*, two or three slices or more of flavedo steeped in hydrosulfite should be clamped together.

Observations on Reduction of Methylene Blue– Frankenthal (7) studied the dehydrogenase activity in the clarified juice of Palestinian orange peel,

TABLE V

Effect of pH on Methylene Blue Reduction Time

Orange	pH	Albedo	Flavedo	Whole rind
		min.	*min*	*min.*
Valencia	8.4	16.5	10.5	12.5
	8 2	15.7	11.4	13.5
	7.9	19 0	12 5	17 2
	6 9	96	52	70
	5.9	600	330	450
Navel	8.2	12.5	8.9	11.5
	7.9	15.5	10.0	14.0
	6.9	60	33.5	43

TABLE VI

Effect of Heat on Methylene Blue Reduction Time for Valencias

pH	Heated juice	Unheated juice
	min	*min.*
7.0	205, 220	56, 54
9.0	34, 35	12.6, 12

and attributed the observed activity to dehydroascorbic acid and its stable oxidation product, diketogulonic acid. Flavedo juice of California oranges was clarified with several types of plant and animal charcoals and norit A gave a juice with highest activity. The methylene blue reduction was found to be catalyzed by light. Since Frankenthal did not define her light conditions, results cannot be directly compared. Direct sunlight caused instantaneous reduction at pH 9. Measurements were made with artificial light at room temperature. With increase in pH, the reduction time was decreased.

The activity was higher in flavedo than in albedo juice, and navel orange juices were more active than those of Valencias (Table V).

Effect of Heat—Flavedo juice was heated to boiling in a water bath, held for 2 minutes, and quickly cooled. After cooling, the solutions were darker yellow and a slight turbidity developed. Times required to decolorize methylene blue are given in Table VI. They are averages of two determinations in each case. At both pH values, the activity was decreased by about two-thirds. This is in agreement with Frankenthal (7). This implies that much of the observed reduction of methylene blue is not enzymatic.

Effect of Cyanide—Frankenthal (7) also reported that the reduction of methylene blue was accelerated by potassium cyanide. To 13.5 ml. of navel flavedo juice, 1.5 ml. of 0.1 M cyanide were added, and the reduction time at pH 9.0 was reduced from 8.5 to 5 minutes, confirming the earlier results (7).

DISCUSSION

Results of the present study appear to show that ascorbase[2] and polyphenolase are absent or non-functioning in the tissues of the orange fruit. Enzymes comparable in many respects with peroxidase, indophenol oxidase (cytochrome oxidase), and dehydrogenase have been shown to be present, though some doubt exists in the case of dehydrogenase, as this may be supplemented by a relatively heat-stable system capable of reducing methylene blue. This has been attributed to ascorbic acid (7).

Measurements of oxygen uptake could not be obtained on tissue extracts or homogenized tissue. Intact tissue was needed. There is considerable inherent variation within oranges selected at random. Comparable variation exists in such fruit with respect to soluble solid content (8). The observation that the highest activity occurs in the flavedo tissue is in agreement with distribution of peroxidase activity (2) and methylene blue reduction (7). Values for Q_{O_2} for flavedo under optimal conditions varied from 1.10 to 2.10 c.mm. per mg. of dry tissue per hour.

The destruction of the activity by heat and the occurrence of an optimum in the pH range 3 to 7 indicate that the observed activity is enzymatic in nature. The optimum pH for the enzyme is on the acid side of neu-

[2] Further study of the situation with respect to ascorbase suggests that this conclusion needs modification. The validity of the ascorbic acid control may be questioned because of the absence of protective factors, of which citric acid may be one (3). The flavedo control, based on average analyses, may contain up to 0.4 mg. of ascorbic acid, and the extent to which this and additional substrate may be protected, or oxidized, depends on the validity of the controls. It appears that the rate of oxidation is independent of the concentration at this and higher levels, owing to saturation of the enzyme. Regardless of this uncertainty, the theoretical maximum for complete oxidation of the ascorbic acid is less than 30 c.mm., while the flavedo control, on the same basis, has an uptake of 140 c.mm.

trality, while autoxidation is greater on the alkaline side. In these studies cyanide inhibition at pH 5.0 is about 50 per cent at 10^{-3} M, and 80 per cent at 10^{-2} M. The respiration of *Nitella* (9) is about 37 per cent inhibited at 10^{-3} M concentration, of barley leaves (10) 65 per cent, of carrots 61 to 72, and of tea leaves (11) 58 to 88. In such tissues as carrot and tea leaf, in which cytochrome oxidase has been reported, inhibition reaches a maximum at 10^{-2} M cyanide, and is by no means complete. This is in contrast with cytochrome oxidase in the absence of other enzymes, when 5×10^{-4} M cyanide produces complete inhibition. Since inhibition increases with increase in cyanide concentration (Fig. 6), the writer favors the suggestion (11) concerning the competition for organic iron between the enzyme and cyanide.

The constant for the ratio of the affinity of the orange enzyme for oxygen and carbon monoxide, namely 14, is close to some of the recorded values, 9 to 12 for carrots (12) and 15 for tea leaf (11).

Cytochrome *b* was observed only in the presence of hydrosulfite, and neither cytochrome *a* nor *c* could be detected.

Frankenthal (7) attributed the dehydrogenase-like activity in the clarified peel juice to the ascorbic acid oxidation products, dehydroascorbic acid and diketogulonic acid, and the results of this investigation with respect to the effect of cyanide, pH, and heat are in general agreement. The ascorbic acid could furnish the basis for dehydrogenase-like activity required for the completion of a respiratory cycle involving cytochrome *b* and a cytochrome-like oxidase.

SUMMARY

Measurements of oxygen uptake have been made on orange tissues by the Warburg technique. The flavedo shows the highest activity with an optimum at about pH 5.0.

The effect of inhibitors and numerous possible added substrates was noted, from which it was concluded that a system involving cytochrome oxidase is responsible for a major part of the oxygen uptake.

The presence of cytochrome *b* was identified spectroscopically by means of its characteristic absorption band at 563 mμ. This was observed only after reduction with hydrosulfite.

Dehydrogenase activity was studied by methylene blue reduction in Thunberg tubes. A somewhat high proportion of this activity (about 30 per cent) appears to be due to a relatively heat-stable system.

spectroscopic work. Thanks are also due to Dr. W. V. Cruess and Dr. L. A. Hohl for their profitable suggestions.

BIBLIOGRAPHY

1. Onslow, M. W , The principles of plant biochemistry, Cambridge, pt. 1, 132 (1931).
2. Davis, W. B., *Am. J. Bot.*, **29**, 252 (1942).
3. Krishnamurthy, P V., and Giri, K. V., *J. Indian Chem. Soc.*, **18**, 7 (1941).
4 Keilin, D., and Hartree, E F., *Proc Roy. Soc. London, Series B*, **127**, 167 (1939).
5. Stotz, E , Sidwell, A. E , Jr , and Hogness, T. R., *J. Biol. Chem.*, **124**, 733 (1938).
6 Stotz, E , A symposium on respiratory enzymes, Madison (1942).
7. Frankenthal, E., *Enzymologia*, **6**, 287 (1939).
8. Bartholomew, E. T., and Sinclair, W. B , *California Citrograph*, **24**, 362 (1939).
9. Ross, E., *Am J. Bot* , **25**, 458 (1938)
10. James, W. O., and Hora, R B., *Ann Bot* , **4**, 107 (1940).
11. Deb, S. B., and Roberts, E A H., *Biochem. J.*, **34**, 1507 (1940).
12. Marsh, A , and Goddard, D. R., *Am J. Bot* , **26**, 767 (1939).

METABOLISM OF THE STEROID HORMONES

IV. CONVERSION OF DESOXYCORTICOSTERONE TO PREGNANEDIOL-3(α), 20(α), IN MAN AND IN THE CHIMPANZEE*

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In a preliminary report (1) from this laboratory, we reported the conversion of desoxycorticosterone to pregnanediol-3(α),20(α) in the ovariectomized chimpanzee. This report deals with the details of the experiments on the chimpanzee as well as similar studies on three men and one woman.

Cuyler *et al.* (2) presented suggestive evidence that the conversion of desoxycorticosterone to pregnanediol-3(α),20(α) takes place in a healthy man. Their data were not conclusive and in subsequent reports they were unable to confirm their original findings either in men or in women (3, 4). Conclusive evidence for this conversion has been reported in rabbits (5, 6).

Administration of Desoxycorticosterone Acetate to Ovariectomized Chimpanzee

Daily doses of 200 mg. of desoxycorticosterone acetate[1] were administered orally for 15 days to an adult ovariectomized chimpanzee. Urine was collected for the 15 days during the hormone administration period and for the following 3 days. A total of 14.9 liters of urine was collected. The urine was extracted in a manner similar to that previously described except for the use of carbon tetrachloride in place of benzene (7). The neutral compounds were separated by means of the Girard-Sandulesco reagent, trimethylacethydrazide ammonium chloride, into the ketonic and non-ketonic fractions. The non-ketonic fraction was separated into the hydroxy and non-hydroxy fractions by means of succinic acid anhydride. The non-ketonic hydroxy fraction was separated further with digitonin.

Those compounds which did not precipitate with digitonin were dissolved in 50 cc. of benzene and adsorbed on a column (10 × 190 mm.) of Brockmann's aluminum oxide. Elution was carried out with benzene and progressively increasing concentrations of ethanol in benzene and finally

with absolute ethanol, as represented in Table I. Crystalline material was found in Fractions 13 to 16 which were eluted with 400 cc. of 1.0 per cent ethanol in benzene. This material after crystallizing several times from 70 per cent ethanol melted[2] at 228–229°. When mixed with an authentic sample of pregnanediol-3(α),20(α), m.p. 229–231°, a melting point of 229–230° was found. The diacetate melted at 175–176° and when mixed with pregnanediol-3(α),20(α) diacetate, m.p. 174–175°, the mixture melted at 174–175°. The yield was 45 mg. of the free compound.

Analysis—$C_{21}H_{36}O_2$ Calculated, C 78.75, H 11 25; found, C 79 04, H 11 70

The mother liquors from Fractions 13 to 16 were combined, evaporated to dryness, dissolved in 25 cc. of benzene, and rechromatographed on a column (10 × 190 mm.) of Brockmann's aluminum oxide. Elutions were

TABLE I

Elution of Compounds Which Did Not Precipitate with Digitonin

Fraction No	Volume of solvent	Solvent	Remarks
	cc		
1	50	Benzene	Trace of colorless oil
2	100	"	" " light brown oil
3–6	135	"	" " colorless oil
7–11	300	0.25% ethanol in benzene	" " " "
12	100	0 5% " " "	" " " "
13–16	400	1.0% " " "	Crystalline material
17	100	2.0% " " "	Trace of colorless oil
18	100	4.0% " " "	" " " "
19	100	8.0% " " "	" " " "
20	100	16 0% " " "	" " brown oil
21	100	Ethanol	" " " "

carried out with benzene and mixtures of ethanol and benzene, of concentrations from 0.5 to 1.0 per cent ethanol, as listed in Table II. The crystalline material from Fractions 5 and 6 (Table II) were combined and acetylated. After recrystallization from dilute ethanol, the material melted at 174–175°. This material was the diacetate of pregnanediol-3(α),20(α). When mixed with an authentic sample of the diacetate, m.p. 174–175°, a melting point of 174–175° was observed. The yield was 43.3 mg. of the diacetate (34.0 mg. of free compound). Thus, the total yield was 79 mg. of pregnanediol-3(α),20(α), representing a 3 per cent conversion.

In a second experiment on the same ovariectomized chimpanzee, twelve daily doses of 100 mg. each of desoxycorticosterone acetate were adminis-

[2] All melting points are uncorrected and were determined with the Fisher-Johns apparatus.

tered orally. A total of 10.8 liters of urine was collected during the period of hormone administration and for the following 4 days. The urinary extracts were prepared and fractionated as described in the first experiment, except that no digitonin precipitation was carried out.

The non-ketonic hydroxy compounds were dissolved in 25 cc. of benzene and adsorbed on a column (10 × 120 mm.) of Brockmann's aluminum oxide. Elutions of the column with a solution of 1 per cent ethanol in benzene yielded crystalline material. Two crystallizations from dilute ethanol yielded a substance melting at 229–230°. The melting point was not depressed when mixed with pregnanediol-3(α),20(α). The diacetate, m.p. 173–174°, was not depressed when mixed with a sample of pregnanediol-3(α),20(α) diacetate, m.p. 174–175°. The yield was 21 mg. of the free compound.

Analysis—$C_{21}H_{36}O_2$. Calculated, C 78.75, H 11.25; found, C 78.66, H 11.31

TABLE II

Elution of Mother Liquors from Fractions 13 to 16

Fraction No.	Volume of solvent	Solvent	Remarks
	cc		
1	25	Benzene	Trace of oil
2	25	"	" " "
3	100	"	" " "
4	200	0 5% ethanol in benzene	Brown oil
5	200	0 75% " " "	Crystalline material
6	100	0.75% " " "	" "
7	100	0.75% " " "	Trace of colorless oil
8	100	1 0% " " "	Nothing

Administration of Desoxycorticosterone Acetate to Three Men and One Woman

Subjects, Methods, Materials—The subjects consisted of three patients suffering from Addison's disease and one from hypogonadism. Two of the patients with Addison's disease were men aged 35 and 33 years, respectively. The third was a 30 year-old woman. The fourth patient was a 28 year-old hypogonadal male.

Desoxycorticosterone acetate was administered orally to the four patients for periods ranging from 5 to 11 days. The total amount of desoxycorticosterone acetate[3] administered per patient, calculated as free compound, ranged from 595 to 1280 mg.

Urine was collected during the period of oral administration of desoxy-

[3] Kindly supplied by Ciba Pharmaceutical Products, Inc., and by the Schering Corporation through the courtesy of Dr. W. H. Stoner.

corticosterone acetate and for the following 2 to 4 days. The combined urine samples were acidified with hydrochloric acid and extracted with carbon tetrachloride as described for the chimpanzee urine. The crude urinary extracts were fractionated by means of sodium hydroxide and the Girard-Sandulesco reagent into the neutral ketonic and neutral non-ketonic fractions.

The neutral non-ketonic fractions were adsorbed on columns of Brockmann's aluminum oxide from solutions of benzene. The adsorbed compounds were eluted from the column by solutions of ethanol in benzene ranging from 0.5 to 3 per cent ethanol in benzene. The principal crops of pregnanediol-3(α),20(α) were eluted by solutions containing 1 per cent ethanol in benzene.

TABLE III

Conversion of Desoxycorticosterone to Pregnanediol-3(α),20(α) in Humans

Patient	Sex	Age	Days administered	Total equivalent of free compound administered	Pregnanediol-3 (α),20 (α) isolated			Conversion
						M p *	M p † of diacetate	
		yrs		mg	mg	°C	°C	per cent
Addison's disease	F	30	11‡	1280	18	232–234	173–174	1 4
" "	M	35	6	618	15	233–235	176	2 4
" "	"	33	5	595	16	230–231	173–174	2 7
Hypogonadal	"	28	7	715	22	233–235	174–176	3 1

* The melting point of an authentic sample of pregnanediol-3(α),20(α) was 231–234°

† The melting point of an authentic sample of pregnanediol-3(α),20(α) diacetate was 173–176°

‡ During the preovulatory phase of the menstrual cycle.

The identity of the isolated products was established on the recrystallized products by means of melting points of the products and their diacetates. Melting points of mixtures of both the free compounds and their diacetates with authentic samples were determined.

The results are summarized in Table III. In each instance, pregnanediol-3(α),20(α) was isolated as a urinary metabolite of desoxycorticosterone. Since the percentage conversion figures are based on the amounts of pregnanediol-3(α),20(α) isolated in crystalline form, the values are necessarily minimal.

The two male patients with Addison's disease showed conversions of 2.4 and 2.7 per cent respectively, while the woman suffering from this disease showed a 1.4 per cent conversion during the preovulatory phase of the menstrual cycle. The hypogonadal male converted desoxycorticosterone to pregnanediol-3(α),20(α) to the extent of 3.1 per cent.

Urine samples collected from the two male patients with Addison's disease before desoxycorticosterone acetate treatment yielded no pregnanediol-3(α),20(α). These studies were carried out on 8 and 5 day specimens from the 33 and 35 year-old males with Addison's disease respectively.

DISCUSSION

The conversion of desoxycorticosterone to pregnanediol-3(α),20(α), which has been demonstrated for man, the chimpanzee, and rabbits, is the first instance in the metabolism of the steroid hormone of the replacement of a hydroxy group (at C_{21}) by a hydrogen atom. The over-all change represents a reduction of the α,β unsaturated ketone in Ring A to the 3-hydroxy group, and the replacement of the hydroxyl group at C_{21} by a hydrogen. The reduction in Ring A is similar to those observed in the transformation of testosterone to androsterone and progesterone to pregnanediol.

A possible mechanism for the changes occurring at C_{20} and C_{21} is presented in the accompanying formulas. The first step involves the reduction of the C_{20} ketone group to the corresponding C_{21} hydroxy group. Thus we have formed a C_{20}-C_{21} glycol which could split out water in either of two ways, forming either a C_{20} or C_{21} enol. For the formation of pregnanediol-3(α),20(α), the C_{21} enol would be the direct intermediate
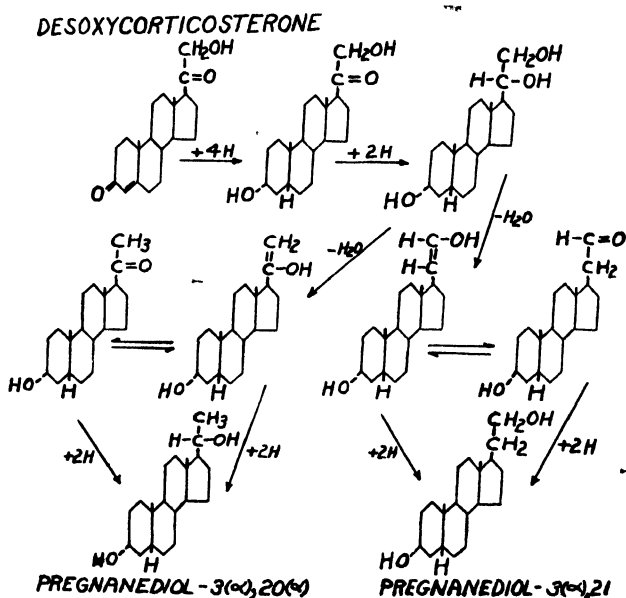
and would form in turn the C_{20} keto tautomer; the reduction of either the enol or keto tautomer would form pregnanediol-3(α),20(α). The C_{21} enol could go through analogous transformations, yielding on reduction pregnanediol-3(α),21.

These findings help to explain the presence of small amounts of pregnanediol-3(α),20(α) in normal men's urine (8) and in ovariectomized women (9).

In the bull, however, the large amount of urinary pregnanediol seems to be associated with the presence of the testis, since the steer excretes no detectable amount of this steroid (10).

SUMMARY

The oral administration of desoxycorticosterone in the form of its acetate to patients with Addison's disease (two men, one woman), a hypogonadal male and an ovariectomized chimpanzee resulted in the urinary excretion of pregnanediol-3(α),20(α).

Conversions of 1.4 to 3.0 per cent of the administered material were observed by direct isolation of the pregnanediol-3(α),20(α).

A possible mechanism of the conversion of desoxycorticosterone to pregnanediol-3(α),20(α) is discussed.

BIBLIOGRAPHY

1. Fish, W. R., Horwitt, B. N , and Dorfman, R. I , *Science*, **97**, 227 (1943).
2. Cuyler, W K , Ashley, C , and Hamblen, E. C., *Endocrinology*, **27**, 177 (1940).
3. Cuyler, W. K , Hirst, D C , Powers, J. M , and Hamblen, E. C , *J. Clin. Endocrinol.*, **2**, 373 (1942)
4. Hamblen, E C., Cuyler, W K , Pattee, C J , and Axelson, G. J., *Endocrinology*, **28**, 306 (1941). ˙
5 Hoffman, M. M., Kazmin, V. E , and Browne, J. S. L., *J. Biol. Chem.*, **147**, 259 (1943).
6. Westphal, U , *Z physiol. Chem* , **273**, 13 (1942).
7. Fish, W. R., and Dorfman, R I , *J Biol. Chem.*, **143**, 15 (1942).
8. Engel, L. L., Thorn, G. W., and Lewis, R. A , *J. Biol. Chem* , **137**, 205 (1941)
9. Hirschmann, H., *J. Biol. Chem.*, **136**, 483 (1940).
10. Marker, R. E., *J. Am. Chem Soc* , **61**, 1287 (1939).

SOME OBSERVATIONS ON THE PHOTOCHEMICAL
DESTRUCTION OF CAROTENE*

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In a recent publication (1) the photochemical destruction of carotene
in the presence of chlorophyll was reported. The present paper presents
some observations concerning the photolysis observed. However, the dis-
cussion is limited by the lack of specific knowledge of the chemical mecha-
nism of the process, since neither the possible intermediates nor end-prod-
ucts have been identified. The experiments described should be considered,
therefore, as attempts to gain an insight into the mechanism of the
reaction

EXPERIMENTAL

In most of the experiments, the mixtures of carotene (90 per cent β- and
10 per cent α-)[1] and 5X chlorophyll[2] in acetone were exposed in test-tubes
to the direct light of a Mazda daylight fluorescent lamp at a distance of
4 inches. The illumination on the reactants was 350 foot candles, as
measured by a Weston illumination meter. The concentrations of carotene
and chlorophyll were determined spectrophotometrically, with the absorp-
tion characteristics at 6500 and 4560 A. In those cases in which a separa-
tion of components was required, as in the experiments involving uranyl ace-
tate or the fluorescent dyes, the carotene was transferred to petroleum
ether and the other reagents removed by washing first with distilled water
and then with 90 per cent methyl alcohol. The carotene solution was dried
by filtering through anhydrous sodium sulfate and the transmittance deter-
mined in a Coleman universal spectrophotometer.

Influence of Oxygen—In an attempt to characterize the reaction, the
question of whether the reaction would proceed in the absence of oxygen
was investigated. Test-tubes containing various carotene-chlorophyll
mixtures in acetone were subjected to the following treatments when ex-
posed to the light source: (1) the tube was kept open to air so that only
dissolved oxygen and that gained by diffusion were available; (2) air was
bubbled through the solution at a rapid rate; (3) pure tank oxygen was

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passed through the mixture; (4) carbon dioxide was bubbled through the solution; (5) nitrogen was passed through the solution as described below.

In the absence of oxygen (CO_2 treatment) no destruction occurred, indicating that oxygen is necessary for the reaction to proceed (Table I). On the other hand, the amount of destruction of carotene was found to be independent of the concentration of oxygen supplied, since identical destruction resulted when oxygen was obtained only by diffusion from the air or

TABLE I

Effect of Oxygen on Photolysis of Carotene in Presence of Chlorophyll (Exposed to Light for 4 Hours)

The values are given in mg

Original		CO₂		Equilibrium with atmosphere		Air		Oxygen	
Chloro-phyll	Carotene	Chloro-phyll	Carotene	Chloro-phyll	Carotene	Chloro-phyll	Carotene	Chloro-phyll	Carotene
*	0 038		0 039				0 017		
1 10	0 025	1 07	0 023			0 65	0 006		
0 44	0 023			0 26	0 001	0 25	0 002	0 30	0 001
0 25	0 007			0 18	0 002			0 17	0 002
0 24	0 038			0 19	0 010	0 19	0 010	0 19	0 009
0 0	0 023	0 0	0 024	0 0	0 022	0 0	0 022	0 0	0.023

* Chlorophyll present but not determined

TABLE II

Effect of Nitrogen on Photolysis of Carotene in Presence of Chlorophyll (Exposed to Light for 4 Hours)

Treatment	Carotene	Chlorophyll
	mg	mg
Original	0 025	0 25
Air	0 004	0 15
Moderate stream of nitrogen	0 010	0.17
Rapid stream of nitrogen	0 023	0.23
Evacuation and flushing with nitrogen	0 020	0 23

when pure tank oxygen was passed through the solution. Furthermore, passing purified nitrogen through pyrogallol and then through the solution stopped the reaction only when a very rapid stream of nitrogen was used or other precautions were taken such as preliminary repeated evacuations and flushing with nitrogen to remove all of the dissolved oxygen. Table II presents some typical results from experiments involving nitrogen.

The conclusions to be drawn from these experiments are (1) oxygen is

required, but only in small amounts; (2) no mass action effect is observed when the concentration of oxygen is increased.

Kinetic Studies—In an attempt to localize the rate-determining step, the order of the reaction was investigated, but because of ignorance of the actual chemical mechanism involved true reaction rates could not be ascertained. The results can only be considered relative either to carotene or to chlorophyll. Aronoff and Mackinney (2) have presented data concerning the destruction of chlorophyll *a* and *b* at different wave-lengths both in the presence and absence of carotene. Their results favor a second order reaction for the photooxidation of the individual chlorophylls alone with a tendency towards a first order reaction in the presence of carotene at a wave-length of 4358 A. The results given in this paper are somewhat at variance with these conclusions, for, although a second order reaction

TABLE III

Reaction Velocity Constants

Mole ratio of chlorophyll to carotene	Chlorophyll, first order constants		Carotene, zero order constants	
	Equation 1	Equations 2 and 3	Equation 4	Equation 2
	min^{-1}	min^{-1}	$\times 10^{-6}$ mole l^{-1} min^{-1}	$\times 10^{-6}$ mole l^{-1} min^{-1}
1 30	−0 0011	−0 0010		
3.51	−0 0012	−0 0013	−0 0141	−0.0133
5 38	−0 0015	−0 0015	−0 0184	−0 0151
7 31	−0 0022	−0 0021	−0 0134	−0 0116
11 90	−0 0022	−0 0024		
13 77	−0 0017	−0 0019	−0 0215	−0 0164
14 87	−0 0018	−0 0018	−0 0185	−0.0200
19 08	−0 0019	−0 0022	−0 0142	−0 0112
29 35	−0 0021	−0 0022		
56 13	−0.0017	−0 0017		

was obtained for chlorophyll alone, good straight lines representing first order plots were obtained for chlorophyll in the presence of carotene at a number of different mole ratios of these two components (Table III)

A series of stoppered tubes containing the desired mixture of chlorophyll and carotene was exposed simultaneously to the light source. Sufficient head room was left in each tube to supply enough air so that the quantity of oxygen was at no time a limiting factor. The tubes were removed at definite time intervals and the reaction stopped by transferring the contents to a 50 ml. volumetric flask and placing it in the dark. The flask was made up to the mark with a minimum exposure to light and read immediately in the colorimeter.

The data were calculated in two ways to afford a check on the accuracy of the results For first order reactions, the formula

$$K_1 = \frac{2.303}{t} \log \frac{C_0}{C} \tag{1}$$

was used where C_0 is the original concentration (moles per liter) of the component and C the concentration at time t (minutes). K_1 is then the first order constant relative to the component.

For confirmation the values of K_1 obtained in this manner were checked by calculating K_1 from the slope of the best straight line through the experimental points as given by

$$\text{Slope} = m = \frac{\Sigma(XY) - \dfrac{\Sigma(X)\Sigma(Y)}{n}}{\Sigma(X^2) - \dfrac{[\Sigma(X)]^2}{n}} \tag{2}$$

which is an application of the law of least squares (3). Therefore,

$$K_1 = 2.303m \tag{3}$$

The values of X and Y used were the experimentally determined values of concentration and time, and not the more ideal values obtained from the plot.

In all experiments, a first order constant with respect to chlorophyll was obtained when carotene was present. Table III gives the K_1 values at a number of different mole ratios of chlorophyll and carotene obtained by both methods of calculation. The values given in the column headed "Equation 1" are the average of five or six individual values obtained at each sampling. A typical set of values is -0.0016, -0.0017, -0.0018, -0.0017, -0.0017, -0.0017, and -0.0017 min.$^{-1}$ obtained at 30 minute intervals.

It is of interest to note that the first order rate constants with respect to chlorophyll are lower as the mole ratio approaches unity, thus indicating a decrease in the rate of the reaction. This observation is in accord with data of Aronoff and Mackinney (2) who found a "protective" action of carotene on the chlorophyll destruction as the carotene concentration was increased to a mole ratio of carotene to chlorophyll of approximately 1:8.

In each case the reaction was zero order with respect to carotene. Straight lines were obtained by plotting concentration against time and good agreement in the rate constants (K_0) were obtained whether calculated from Equation 2 or from

$$-\frac{\Delta C}{\Delta t} = K_0 \tag{4}$$

The results are given in Table III. The data in the column headed "Equation 4" are the average of five determinations. A typical set of

values determined at 30 minute intervals are −0.011, −0.011, −0.016, −0.013; average = −0.013 × 10⁻⁶ mole liter⁻¹ min.⁻¹.

Since the reaction is independent of the concentration of oxygen and of carotene (zero order), it can be concluded that the rate-determining step involves the photochemical activation of chlorophyll, which in turn results in the oxidation of carotene.

Two possibilities exist as to the mechanism of the reaction. First, there may be the formation of a complex between the chlorophyll and the carotene molecules. However, the kinetic measurements mitigate against this idea, since the reaction is apparently first order with respect to chlorophyll and zero order with respect to carotene. Furthermore, Aronoff and Mackinney (2) could find no spectrophotometric evidence for such a compound formation.

The second and most plausible possibility is the activation of oxygen through the irradiation of chlorophyll to form either a pigment peroxide or acceptor peroxide. According to Gaffron (4) the function of the irradiated chlorophyll in similar reactions is to produce an acceptor peroxide which through autoxidation results in the photolysis of the acceptor. Accordingly, based on Gaffron's hypothesis, the mechanism of the reaction can be briefly postulated as follows:

Light + oxygen + chlorophyll + carotene → carotene-peroxide →˙ products of
carotene photolysis
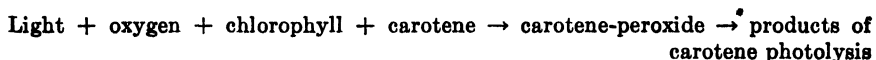
Any further discussion is not warranted by the data, and must necessarily wait until the products of the reaction are identified.

Effect of Other Photodynamic Materials—Since eosin, fluorescein, methylene blue, and the uranyl ion have proved to be photochemically active in a great number of reactions, the influence of these photodynamic materials on carotene was investigated. Except for fluorescein, they have all produced photolysis of carotene under the experimental conditions employed although at different rates. The uranyl ion supplied as uranyl acetate posed some special problems and will be discussed separately.

Fig. 1 indicates the relative rates of reaction. Each point represents the relative amount of carotene destroyed at a given time interval. The dyes were each at 50 × 10⁻⁶ mole per liter at the start and the initial concentration of carotene was 6.3 × 10⁻⁶ mole per liter.

It is evident from the graph that methylene blue is much more effective in producing the photolysis of carotene than is chlorophyll. Eosin is somewhat less effective, while fluorescein had no effect at all.

A parallel reaction involving methylene blue is described by Bernheim and Dixon (5, 6) in which xanthine oxidase is destroyed by irradiating the enzyme in the presence of methylene blue and a trace of oxygen. The

reaction proceeds through the formation of peroxide, thus suggesting a similarity of the photolysis of carotene in the presence of chlorophyll. Eosin has previously been shown to resemble chlorophyll in its photochemical action although it is less active, as is indicated above (7).   The failure of fluorescein to effect the photolysis of carotene cannot be explained from the available data.

The uranyl ion, supplied as uranyl acetate, produced somewhat different results.   Because of the insolubility of uranyl acetate in acetone, all experiments were carried out in 50 per cent acetone in which the uranium salt was soluble

As indicated in Fig. 1, at the concentration of the uranyl ion used (50 $\times$ 10^{-6} mole per liter), the reaction proceeded more slowly than in the presence
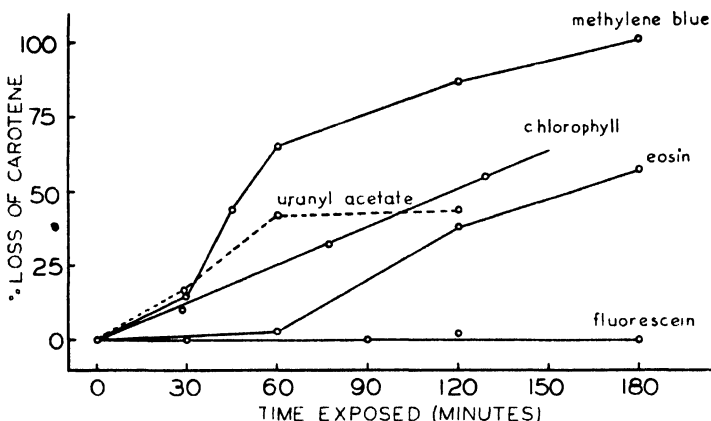
Fig 1. Relative rate of the photolysis of carotene in the presence of methylene blue, chlorophyll, eosin, uranyl acetate, and fluorescein

of methylene blue, and after 60 minutes reached a maximum.   However, upon further experimentation, it was observed that under the proper concentration of uranyl acetate, the reaction proceeded with surprising speed, destroying, at the optimum concentration, over 80 per cent of the carotene in 30 minutes.

The optimum rate for photolysis of carotene occurred when the uranyl-carotene ratio was approximately 50:1.   Below this ratio, the rate falls off rapidly, producing only a 20 per cent destruction of carotene at a mole ratio of 25:1.   Above the optimum ratio the rate again falls off, but more slowly.   At a mole ratio of 100:1 the destruction of carotene decreased to approximately 50 per cent of the initial concentration.   No explanation of these observations can be given at present.

SUMMARY

1. Oxygen is required for the photolysis of carotene in the presence of chlorophyll. The evidence indicates, however, that only traces of oxygen are required and that no mass action effect is produced as the concentration of oxygen is increased.

2. Kinetic studies indicate that the reaction is first order with respect to chlorophyll and zero order with respect to carotene.

3. The rate-determining step at constant light intensity probably involves the photoreactivity of chlorophyll to produce an acceptor peroxide (carotene peroxide) which results in the destruction of carotene through autoxidation.

4. Methylene blue and eosin also induced the photochemical destruction of carotene. Methylene blue was more effective than chlorophyll and eosin less effective. Fluorescein had no effect.

5. The uranyl ion supplied as uranyl acetate was extremely active as a photolytic agent at a mole ratio of uranyl to carotene of approximately 50:1 Below this ratio, the rate dropped off rapidly and above this ratio the rate decreased more slowly.

BIBLIOGRAPHY

1 Pepkowitz, L P , *J Biol Chem* , **149,** 465 (1943)
2 Aronoff, S , and Mackinney, G , *J Am Chem Soc* , **65,** 956 (1943)
3 Ford, W B , A first course in the differential and integral calculus, New York, 343 (1928)
4 Gaffron, H , *Ber chem Ges* , **60 B,** 2229 (1927), *Biochem. Z* , **264,** 251 (1933)
5 Bernheim, F , and Dixon, M , *Biochem J* , **22,** 113 (1928)
6 Bodansky, M , Introduction to physiological chemistry, New York, 3rd edition, 147 (1934).
7 Meyer, K , *J Biol Chem* , **103,** 597 (1933).

THE RED PIGMENT OF LEGUMINOUS ROOT NODULES*

BY R. H. BURRIS AND ERWIN HAAS

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and the Department of Chemistry, University of Chicago, Chicago)*

Pietz (1) observed in root nodules of horse-beans (*Vicia faba*) a red pigment capable of reversible oxidation and reduction depending on the presence of oxygen. He concluded that this substance was identical with 5,6-quinone-2,3-dihydroindole-2-carboxylic acid, the red intermediate, obtained by Raper (2) upon enzymatic oxidation of tyrosine. Kubo (3) has described a method for the extraction and fractionation of the red pigment from leguminous root nodules and has reported, contrary to Pietz, that it is a hemoglobin-like substance. He used a crude extract of soy beans, and his observations were limited to the visible range of the spectrum without consideration of the blue region of the spectrum which shows absorption bands typical for hemin compounds. His description of two absorption maxima at 575 and 540 $m\mu$ for the oxidized form and one maximum at 555 $m\mu$ for the reduced form was based on observations made with a micro spectroscope. The red pigment in the impure extract was present in the reduced state, but temporary reversible oxygenation occurred during the time the solution was shaken with air. The experimental data obtained here with the substance purified by precipitation are in disagreement with the classification of this chromoprotein either as a quinone- or a hemoglobin-like compound.

The red pigment of root nodules of cow-peas (*Vigna sinensis*) was purified by the method of Kubo. The material obtained by fractionation with 0.50- to 0.75-fold saturation with respect to ammonium sulfate was dialyzed against water and finally dissolved in 0.05 M phosphate buffer of pH 7.1. The relative absorption spectrum of this solution as shown in Fig. 1 was determined by photoelectric spectrophotometry (4), light absorption (log I_0/I) being plotted as a function of the wave-length. The absorption line in the blue region, which is characteristic for hemin compounds, distinguishes this substance from the quinoid intermediate proposed by Pietz, since Mazza and Stolfi (5) have found the absorption maximum of the latter compound at 539 $m\mu$. The spectrum of the oxidized form exhibits only one band in the visible range at 530 $m\mu$ in contrast to the hemoglobin-like double band reported by Kubo. After deoxygenation of the solution the spectrum of the oxidized form remains unchanged, indicating true oxidation

and not mere oxygenation of the red pigment.   Reduction by hydrosulfite is accompanied by a considerable change of the absorption spectrum, as indicated in Fig. 1.   Like other hemin compounds the band in the blue region of this pigment is shifted to a longer wave-length upon reduction. Contrary to the process reported in hemin-containing proteins, reduction is here followed by an unusual decrease in light absorption.   In summary, the spectrum definitely shows that the pigment is a hemin compound but not a quinoid compound.   The method of preparation, which includes
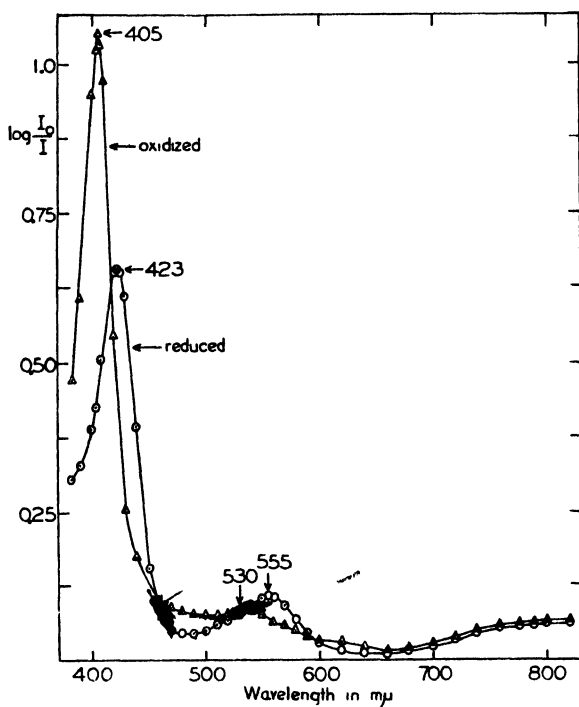
Fig. 1   Spectrum of the pigment of root nodules

ammonium sulfate precipitation in a narrow range and subsequent dialysis, indicates that the substance is a compound of high molecular weight, most probably a protein.   The fact that it is precipitable by trichloroacetic acid and by heat and has a nitrogen content of 16.0 per cent is further indicative of its protein nature.   Its behavior towards oxygen indicates that it is not a hemoglobin-like substance.

The regular occurrence of a red pigment in root nodules suggests a possible rôle for it in the respiration of this tissue; Kubo has reported that it stimulates the respiration of *Rhizobium japonicum*.   Attempts were made

here to determine the possibility of an interaction between the red pigment and reconstructed diphosphopyridine nucleotide or triphosphopyridine nucleotide systems. Measurements were made at 430 mμ because the greatest difference between the absorption of the oxidized and reduced compound occurs at this wave-length. No reduction of the red pigment could be achieved by dihydrodiphosphopyridine nucleotide or dihydrotriphosphopyridine nucleotide. The pigment remained in its oxidized form even after simultaneous addition of the reduced coenzymes together with a "Lebedev" juice containing high concentrations of cytochrome reductase, alcohol dehydrogenase, yellow enzymes, etc. No evidence has been found to demonstrate any functional rôle of the pigment in tissue oxidations by way of the diphosphopyridine nucleotide or triphosphopyridine nucleotide systems.

BIBLIOGRAPHY

1 Pietz, J , *Zentr. Bakt , 2 Abt* , **99,** 1 (1938)
2 Raper, H S , in Nord, F F , and Weidenhagen, R , Ergebnisse der Enzymforschung, Leipzig, **1,** 270 (1932)
3 Kubo, H , *Acta phytochim* , Japan, **11,** 195 (1939)
4. Hogness, T. R , Zscheile, F P , Jr , and Sidwell, A E , Jr , *J. Phys. Chem.*, **41,** 379 (1937)
5 Mazza, F P , and Stolfi, G , *Arch sc. biol* , **16,** 183 (1931)

STUDIES IN CARBOHYDRATE METABOLISM

I. THE RATE OF TURNOVER OF LIVER AND CARCASS GLYCOGEN, STUDIED WITH THE AID OF DEUTERIUM*

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During the synthesis of glycogen in the animal body it has been shown that stably bound deuterium may be introduced if the body fluids are enriched with heavy water (1, 2). This fact makes possible the determination of the rate of turnover of glycogen in normal animals at constant weight. We have therefore investigated the rates of turnover of both liver and carcass glycogen by the expedient of enriching the body fluids of rats with D_2O, maintaining a uniform level of D_2O in the body fluids for varying periods of time, then killing the animals, and isolating glycogen from the liver and carcass. Comparison was made between the deuterium concentration in these samples and that in the body water.

In all the experiments to be described, adult female rats were used. They were kept on the experimental diet, which contained 60 per cent glucose and no fat, until constant body weight was attained. Sufficient D_2O was then injected subcutaneously to raise the deuterium level of the body fluids to approximately 2 per cent, and at the same time the drinking water was replaced by 3.5 per cent D_2O. Animals were killed at intervals from 3 hours to 16 days after administration of heavy water was started. Glycogen was isolated from the liver and carcass, and, for comparative study, the liver fatty acids were also obtained.

From the results in Table I, it is evident that the deuterium concentration in liver and carcass glycogen has increased with time. It may be shown that if the total quantity of any body constituent remains constant, if the amount replaced per unit time is constant, and if the synthetic process involves the uptake of isotope from a reservoir of constant concentration, the equation

$$\ln \frac{i_{max.}}{i_{max.} - i} = kt \tag{1}$$

represents the relationship between the isotope concentration of that body constituent and time. Here, i is the isotope concentration at time t;

$i_{max.}$, the isotope concentration at infinite time; and k, the fraction of the constituent replaced per unit time. In the present series of experiments the three conditions necessary for the application of this equation have been approximated in so far as the body weight, diet, and level of deuterium in the body fluids were constant during the period of observation. The quantities of glycogen recovered from each of the six animals did not vary significantly.

TABLE I

Uptake of Deuterium from Body Water

Six adult rats were maintained at constant weight on a high carbohydrate diet The body fluids were enriched with D_2O for varying periods of time, the rats then killed, and liver and carcass glycogen, as well as liver fatty acids, analyzed for deuterium　The values are reported as (a) the actual analytical figures, and (b) recalculated for 100 atom per cent deuterium in the body fluid.

Duration	Concentration of deuterium						
	Analytical figures				Recalculated values		
	Body water	Liver fatty acids	Liver glycogen	Carcass glycogen	Liver fatty acids	Liver glycogen	Carcass glycogen
days	*atom per cent D*	*atom per cent D*	*atom per cent D*	*atom per cent D*	*per cent of body water*	*per cent of body water*	*per cent of body water*
0 125	2 23	0 120	0 013	0 037	5 4	0 6	1.7
1	2 45	0 379	0 480	0 074	15 5	19 6	3 0
2	2 32	0 508	0 380	0 141	21 9	16 4	6.1
4	2 22	0 618	0 523	0 212	27 8	23 6	9 6
8	1 94*	0 695	0 560	0 386	35 8	28 8	19 9
16	1 78*	0 674	0 514	0 432	37 8	28 9	24 3

* In these experiments of long duration, somewhat lower concentrations of D_2O were employed, in the interests of economy

In order to evaluate k, an extrapolated value for i_{max} has been obtained from the equation

$$i_{max} = \frac{i_1^2}{2i_1 - i_2} \tag{2}$$

where i_1 is the isotope concentration at time t_1 and i_2 the isotope concentration at time $t_2 = 2t_1$. The isotope values for the 8 and 16 day experiments were employed in this extrapolation. The values of the function $\ln i_{max}/(i_{max} - i)$ have been plotted against time and the best straight lines fitted to the resulting points by the method of least squares (Fig. 1). The slopes of these lines are equal to k in each case. The half life of each substance has also been calculated from the equation

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \tag{3}$$

From these results, given in Table II, the quantity of glycogen turned over per rat per day may be estimated. The mean weight of liver glycogen recovered from the six animals was 547 ± 68 mg., and of carcass glycogen, 331 ± 41 mg. The weight of glycogen turned over per rat per day was therefore $0.68 \times 547 = 372$ mg. in the liver, and $0.19 \times 331 = 63$ mg. in the carcass, or about 435 mg. in all. During the experimental period the rats consumed approximately 23 gm. of diet, or about 14 gm. of glucose per day. Of this, only 0.44 gm., or about 3 per cent, was handled by way of glycogen.
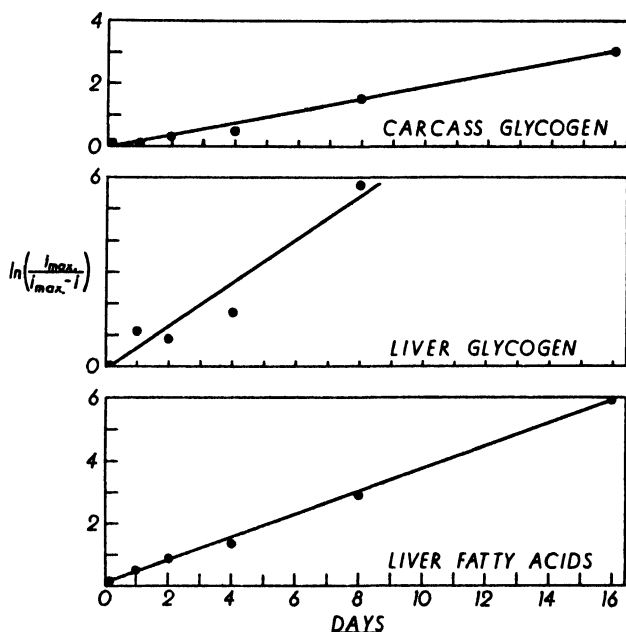
Fig. 1 Uptake of deuterium from the body water The values of the function $\ln i_{max}/(i_{max} - i)$ have been plotted against time and the best straight lines determined by the method of least squares.

It is of interest to speculate on the metabolic pathway of the remaining 97 per cent of the ingested glucose. A portion may well have been burned directly, without preliminary glycogenesis. Another portion was undoubtedly converted to fatty acid. The magnitude of this fraction may be estimated from the value for k for the body fatty acids of rats on a high carbohydrate diet, calculated from the results of Bernhard and Bullet (3) as 0.077 day^{-1}. As our animals contained about 25 gm. of fatty acids, approximately $0.077 \times 25 = 1.9$ gm. of fatty acids were burned each day. As the animals were on a fat-free diet and at constant weight, it must be

presumed that an equal quantity of fatty acids, newly synthesized from glucose, was deposited. If it is further assumed that glucose is initially split to 3-carbon fragments, and that only 2 of these 3 carbon atoms are directly available for fatty acid synthesis, it may be calculated that at least 5 gm. of glucose were needed to synthesize 1.9 gm. of fatty acids. In other words, in our rats on a high carbohydrate, fat-free diet more than 10 times as much of the dietary glucose was used each day in the synthesis of fatty acids as was consumed in the synthesis of glycogen. From our results it must be concluded that, at least in the well nourished rat on a high carbohydrate diet, glycogen represents a quantitatively minor pathway of glucose metabolism.

The maximal isotope concentrations in both the liver and carcass glycogen, achieved only in infinite time under our experimental conditions, lay between 25 and 30 per cent of the deuterium concentration in the body

TABLE II

Rate of Turnover of Liver Glycogen, Carcass Glycogen, and Liver Fatty Acids

The fraction of each constituent replaced per day, k, and the half life of each constituent, $t_{\frac{1}{2}}$, have been calculated from the figures in Table I   The maximal isotope concentrations, i_{max}, expressed as per cent of the isotope concentration of the body water, are also given

Body constituent	k	$t_{\frac{1}{2}}$	i_{max}
	day^{-1}	days	per cent of body water
Liver fatty acids	0 37	1.9	37.9
" glycogen	0 68	1.0	28 9
Carcass glycogen	0 19	3 6	25 5

water. We consider these maxima to be in good part the results of the conditions selected, for, as will be shown in subsequent publications, these values may be exceeded either by change of diet or by disease. The simplest explanation of these values is that glycogen is made by two types of processes in the animal. The one process, the more or less direct combination of dietary hexose molecules, would not be expected to introduce much deuterium into the resulting glycogen. The second process would be glycogenesis from fragments smaller than hexose. In such a process it is conceivable that each H atom in the resultant glycogen was, at some stage, either in equilibrium with or derived from the body water. Such a process would result in a glycogen of the same isotopic composition as the body water. In the course of isolation and purification of glycogen, however, a fraction of the labeled H is necessarily lost. From consideration of its formula, and from a report by Ussing (1), about 3 out of every 10

hydrogen atoms of glycogen would be expected to be immediately exchangeable with water. We have determined the fraction of immediately exchangeable H in glycogen by preparing a solution of glycogen in heavy water, evaporating to dryness, and analyzing the residual glycogen for deuterium. The deuterium concentration of this glycogen was 34 per cent of that of the water, indicating that 66 per cent of the hydrogen in glycogen is stably bound. Therefore, even if a sample of glycogen in the animal were of the same isotopic composition as the body water, the highest deuterium concentration that could be expected in this glycogen after purification by repeated precipitation from normal water would be 66 per cent of that in the body fluids. We interpret the fact that our values for $i_{max.}$ (Table II) were less than half of this theoretical maximum to mean that in the synthesis of liver and carcass glycogen both types of processes have contributed, and to approximately the same extent in both cases.

EXPERIMENTAL

The diet used throughout consisted of glucose monohydrate 60 parts, casein (Labco, vitamin-free) 22 parts, yeast powder 6 parts, salt mixture (4) 6 parts, and roughage (Celluration) 6 parts. Adult female rats of the Sherman strain, weighing from 267 to 300 gm., were kept in individual cages and offered the diet and water *ad libitum*. When constant weight was attained, deuterium oxide was administered by injection and in the drinking water, as described above. The animals were killed by a blow on the head and immediately eviscerated. The livers were placed in hot 30 per cent aqueous KOH and heating was continued until complete disintegration had taken place. The eviscerated carcasses were minced and treated in the same manner as the livers. The total time consumed between killing of the rat and immersion of the tissues in alkali was less than 10 minutes.

The same procedure was employed for the isolation of glycogen from liver and from carcass. The alkaline digest was filtered through glass wool and 1.2 volumes of 95 per cent ethanol were added. The precipitate was removed by centrifugation and washed with 60 per cent ethanol. Fatty acids were isolated from the supernatant solution. The precipitated glycogen was extracted with three portions of 10 per cent trichloroacetic acid and reprecipitated from the combined extracts by addition of 1.2 volumes of ethanol. The glycogen was further purified by two successive precipitations from distilled water. As glycogen precipitates poorly in the complete absence of electrolytes, it was found advantageous to add a crystal of lithium bromide at the time of the last alcoholic precipitation. This salt was selected because of its solubility in alcohol. The final product was washed with increasing concentrations of ethanol, finally with acetone, and

was dried *in vacuo* over P_2O_5 at room temperature. It contained less than 1 per cent of ash, and less than 0.05 per cent of N. After acid hydrolysis, it yielded from 96 to 102 per cent of the glucose required by theory.

In order to demonstrate that none of the otherwise stably bound hydrogen atoms in glycogen were rendered labile by concentrated alkali, normal glycogen was refluxed for 18 hours with 30 per cent KOH in 3 per cent D_2O. The glycogen, isolated and purified as described above, contained no deuterium.

To ascertain the per cent of readily exchangeable hydrogen in glycogen, a 10 per cent solution of normal glycogen was prepared in approximately 3 per cent D_2O and kept at room temperature for 24 hours. The solution was frozen, and water removed by sublimation at high vacuum. The collected water contained 2.72 atom per cent deuterium, and the dried glycogen, 0.913 atom per cent deuterium. Thus, 33.5 per cent of all the hydrogen atoms of glycogen had undergone exchange.

Deuterium analyses were carried out by the falling drop technique (5).

SUMMARY

From observations of the uptake of stably bound deuterium into liver and carcass glycogen in adult rats at constant weight, when the body fluid was maintained enriched with D_2O, the rate of turnover of these substances has been calculated. The half life of liver glycogen was found to be about 1 day, that of carcass glycogen about 3.6 days.

It was calculated that on the high carbohydrate diet employed only about 3 per cent of the dietary glucose was handled by way of glycogen and that at least 10 times as much of the dietary glucose was used to synthesize fatty acids as was used to synthesize glycogen.

Evidence has been presented to support the view that both liver and carcass glycogen are formed in part from dietary glucose and in part from smaller units.

BIBLIOGRAPHY

1. Ussing, H H , *Skand Arch. Physiol* , **77,** 85 (1937).
2. Boxer, G E , and Stetten, D , Jr , *J. Biol. Chem* , **153,** 617 (1944)
3. Bernhard, K , and Bullet, F , *Helv chim acta,* **26,** 1185 (1943)
4. Osborne, T B , and Mendel, L B , *J. Biol Chem* , **15,** 311 (1913)
5. Keston, A S , Rittenberg, D , and Schoenheimer, R , *J Biol. Chem.,* **122,** 227 (1937–38)

STUDIES IN CARBOHYDRATE METABOLISM

II THE GLYCOGENIC RESPONSE TO GLUCOSE AND LACTATE IN THE PREVIOUSLY FASTED RAT*

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In Paper I of this series a study of the rate of turnover of liver and carcass glycogen in well nourished rats at constant weight on a high carbohydrate diet has been reported (1). It was concluded that under these conditions the half life of liver glycogen was about 1 day, that of carcass glycogen about 4 days. These conclusions were arrived at by determination of the rate of increase of deuterium concentration in glycogen when the body fluids of rats were enriched with heavy water, and it was noted that in these experiments the deuterium concentrations of both liver and carcass glycogen approached asymptotically values of 25 to 30 per cent of that in the body water.

This finding has been provisionally interpreted to indicate that both liver and carcass glycogen are synthesized by two types of processes. The one process, direct coupling of dietary glucose molecules, would introduce little isotope into glycogen, whereas the other, glycogenesis from fragments smaller than hexose, might conceivably result in glycogen of the same isotopic composition as the body water. We have previously stated the reasons for our belief that, if the hydrogen in glycogen in the animal did indeed contain the same concentration of deuterium as the body water, it would contain only 66 per cent as much deuterium as the body water after the exchangeable deuterium had been lost during isolation.

Cori (2–4) has shown that the administration of glucose or lactate to a previously fasted rat is promptly followed by the synthesis of new glycogen and Hastings and collaborators (5–9), employing C^{11}, have investigated the sources of the carbon in the glycogen synthesized under these conditions. In the present experiment we have set out to determine the deuterium concentration of liver and carcass glycogen synthesized in the previously fasted rat in response to administration of glucose or lactate, when the body fluids were enriched with D_2O. In each of the experiments to be discussed, six adult female rats were first fasted for 24 hours. Sufficient D_2O was then injected subcutaneously to bring the level of deu-

terium in the body water to a value of approximately 1.2 to 1.3 per cent. Feedings were given by stomach tube 1 hour after injection, glucose in one case, and *dl*-lactic acid, half neutralized, in another. A third group of animals received no feeding. 4 hours after the injection of D_2O, the animals were killed, glycogen as well as fatty acids isolated from the livers and carcasses, and their deuterium concentrations compared with those of the body water.

The validity of the procedure of administering all the isotope at one time in the form of a single injection of D_2O was confirmed by the actual determination of the rate of decrease of deuterium concentration in the body fluids following such an injection. The concentration of deuterium in the
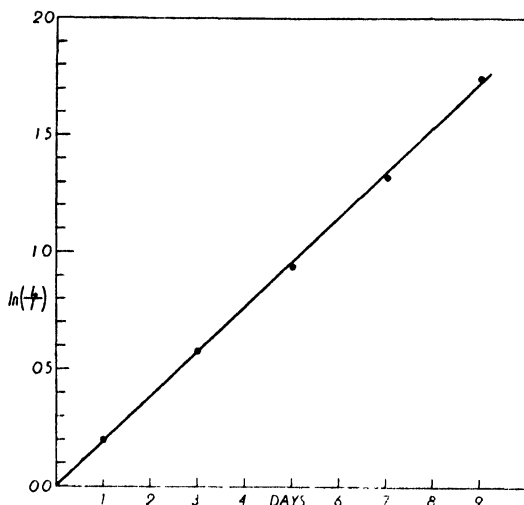
FIG. 1. Decrease in deuterium concentration of urine water. The values of the function $\ln (i_0/i)$ have been plotted against time

urine water was determined at intervals after an injection of D_2O, and the fraction of hydrogen in water replaced per day, k, was evaluated from the solution of the equation

$$kt = \ln (i_0/i) \tag{1}$$

where i_0 is the isotope concentration at zero time and i the isotope concentration at time t thereafter (Fig. 1). The value of k, under our experimental conditions, was found to be 0.192 day^{-1} and the half time of replacement of water molecules, with respect to their hydrogen content, 3.62 days. During the 4 hour period of the short time experiments, the deuterium concentration of the body fluids should not have decreased by more than 3.5 per cent.

Before we discuss the significance of the values in Table I, it should be pointed out that in well nourished rats on a high carbohydrate diet the deuterium concentrations of liver and carcass glycogen had reached values of 0.6 and 1.7 per cent respectively of that in the body water 3 hours after the injection of D_2O (1). The uptake of deuterium into glycogen is a very much more rapid process in the previously fasted animal than in the well nourished animal.

In the animals that were simply fasted, the isotope concentration of the liver glycogen had risen to 25, of the carcass glycogen to 12 per cent of that in the body water. We cannot estimate the maximal values of these isotope concentrations, and are therefore unable to calculate the rate constants. To have reached these levels, however, at least 38 per cent of all the liver glycogen and 18 per cent of all the carcass glycogen must

TABLE I

Uptake of Deuterium from Body Water

Deuterium oxide was injected subcutaneously into three groups of six adult rats each that had previously been fasted for 24 hours. To the first group no food was given, but the second and third groups were given glucose and lactate respectively by stomach tube 1 hour after the injection. The rats were killed 4 hours after injection.

Feeding	Liver glycogen	Carcass glycogen	Body water	Liver glycogen	Carcass glycogen	Liver fatty acids	Depot fatty acids	Liver glycogen	Carcass glycogen	Liver fatty acids	Depot fatty acids
	per cent of liver	*per cent of carcass*	*atom per cent D*	*atom per cent D*	*atom per cent D*	*atom per cent D*	*atom per cent D*	*per cent of body water*	*per cent of body water*	*per cent of body water*	*per cent of body water*
None	0.04	0 011	1 19	0 30	0 142	0 003	0 014	25 1	11 9	0.3	1.2
Glucose	0 82		1 29	0 49	0 183	0 016	0 011	38 1	14.2	1.2	0 9
Lactate	0 21	0 012	1 28	0 73	0 198	0 022	0 028	57 0	15 4	1.7	2.2

have been synthesized in the last 4 hours of life. In the same time interval, the well nourished rats would have synthesized only 11 per cent of their liver glycogen and 3 per cent of their carcass glycogen.

When glucose was given to previously fasted rats 3 hours before death, the quantity of glycogen recovered from the livers was 17.5 times greater than in the fasted control animals. This material was but slightly diluted by preexisting non-isotopic glycogen and its deuterium concentration must therefore be a close approach to the maximal value for glycogen synthesized in the livers of the previously depleted animals when glucose is fed. The isotope value, 38 per cent of the deuterium concentration in the body water, is appreciably higher than the maximal value achieved only at infinite time in well nourished animals, 29 per cent (1). This leads to the conclusion that a greater proportion of the hydrogen atoms of

glycogen had been derived from the body water in the previously fasted animal than in the well nourished animal. The most plausible explanation for this finding is that the preceding period of fasting results in a preference of glycogenesis from fragments smaller than hexose rather than from dietary glucose directly. This is in accord with the observation of Vennesland, Solomon, Buchanan, and Hastings (8) that glycogen synthesized in response to glucose administration to previously fasted rats contained C^{11} if $NaHC^{11}O_3$ were simultaneously injected.

The quantity of glycogen recovered from the livers of the rats that were given lactate was more than 4 times that in the fasted control rats. The isotope concentration of this glycogen was 57 per cent of that in the body water. We have previously shown that 34 per cent of all the hydrogen in glycogen is exchanged on solution in water. It follows that, if a sample of glycogen, after purification by repeated precipitation from water, has 66 per cent as high a concentration of deuterium as the body water of the animal from which it was obtained, the glycogen must *in vivo* have been of the same isotopic composition as the body water. The analytical value in the present experiment, 57 per cent, is a very fair approximation to this theoretical maximum. It indicates that during glycogenesis from dietary lactic acid essentially all of the hydrogen in the new glycogen is either derived from or exchangeable with the body water at some stage.

From the fact that a sample of glycogen of almost the predicted maximal deuterium concentration has been obtained, it may further be concluded that, under the conditions of our isolation procedure, the carbon-bound hydrogens of glycogen are stable.

It is now possible to interpret earlier experiments in which liver glycogen was isolated from rats receiving D_2O kept on complete and thiamine-deficient diets (10). After 5 days on D_2O, the glycogen from the livers of rats on the complete diet contained 19, on the thiamine-deficient diet, 41 per cent of the isotope concentration in the body water. The process of glycogenesis in the thiamine-deficient rat favored the introduction of more deuterium from the body water than in the normal control animal. This is taken to mean that in thiamine deficiency glycogenesis proceeds preponderantly from fragments smaller than hexose, possibly chiefly from pyruvate.

Of the carcass glycogen samples isolated, it may be pointed out that their deuterium concentrations are from one-half to one-fourth those of the corresponding liver glycogens. The rate of incorporation of deuterium into carcass glycogen was much more rapid in these fasted animals than it was in the well nourished rats. Since fasting does not deplete the muscle of glycogen to the same extent as the liver (2), the newly synthesized deuterio glycogen must be assumed to be diluted by non-isotopic glycogen

initially present in the muscle. The isotope concentrations found in the carcass glycogen samples cannot, therefore, be considered as maximal values.

The determination of the rate of turnover of body water was carried out on an adult female rat of the Sherman strain. The rat was kept at constant weight, 300 gm., on a diet previously described (1). Food and water were given *ad libitum*. 7 cc. of 99.5 per cent D_2O were injected subcutaneously and urine samples were obtained after 1, 3, 5, 7, and 9 days. Each sample represented the urine output over a 3 hour period, from $1\frac{1}{2}$ hours before to $1\frac{1}{2}$ hours after the time stated. Water was distilled off and analyzed for deuterium. The best values for k and i_0 were obtained from the isotope analyses by the method of least squares. The half life, $t_\frac{1}{2}$, was then calculated from k. The results are given graphically in Fig. 1.

$$k = 0\ 1917 \pm 0\ 0011\ \text{day}^{-1}$$
$$t_\frac{1}{2} = 3\ 62\ \text{days}$$
$$i_0 = 2\ 94\ \text{atom per cent D}$$

The animals used in the glycogen experiments were female adult rats averaging 200 gm. in weight. They were taken off the stock diet 24 hours before injection of D_2O, but water was offered *ad libitum*. The rats lost from 8 to 9 per cent of their body weight during this fast. 1 cc. of 99.5 per cent D_2O was injected hypodermically per 100 gm. of body weight. Tube feedings were carried out by the technique described by Levin (11). 250 mg. of glucose in 1 25 cc. of water were administered to each rat of one group and 400 mg. of *dl*-lactic acid, half neutralized with NaOH, in 1.25 cc. of water, were given each rat of another group. The animals were killed exactly 4 hours after injection of D_2O, and their bodies worked up as described in the previous report (1).

SUMMARY

The uptake of deuterium in the liver and carcass glycogen of the rat, after injection of D_2O, has been studied in fasted animals and in animals fed glucose or lactate after previous fasting.

The appearance of deuterium in glycogen proves to be a very much more rapid process in the fasted than in the well nourished rat.

Previous fasting favors a process of glycogenesis in response to ingested glucose that results in glycogen rich in isotope. This is interpreted as indicating glycogenesis from fragments smaller than hexose rather than from dietary glucose directly.

Glycogenesis from dietary lactate gives liver glycogen of nearly the maximum deuterium concentration theoretically possible, indicating that during synthesis all the hydrogen is derived from or exchangeable with body water.

Additional evidence for the stability of the carbon-bound hydrogen in glycogen is presented.

The half time of replacement of the hydrogen in body water in the rat, under our experimental conditions, was incidentally determined to be 3.6 days.

BIBLIOGRAPHY

1. Stetten, D , Jr , and Boxer, G. E., *J. Biol. Chem.*, **155,** 231 (1944).
2. Cori, C. F., and Cori, G T , *J. Biol. Chem.*, **70,** 557 (1926).
3. Cori, C. F., *J. Biol. Chem* , **70,** 577 (1926).
4. Cori, C. F , and Cori, G T , *J. Biol. Chem.*, **81,** 389 (1929).
5. Conant, J B , Cramer, R. D., Hastings, A. B., Klemperer, F. W., Solomon, A. K , and Vennesland, B , *J. Biol Chem* , **137,** 557 (1941)
6. Solomon, A K., Vennesland, B , Klemperer, F. W , Buchanan, J. M , and Hastings, A B., *J Biol Chem.*, **140,** 171 (1941).
7. Vennesland, B , Solomon, A K., Buchanan, J. M , Cramer, R D , and Hastings, A. B., *J Biol Chem* , **142,** 371 (1942).
8. Vennesland, B , Solomon, A. K., Buchanan, J. M , and Hastings, A B , *J. Biol Chem* , **142,** 379 (1942).
9. Buchanan, J M , Hastings, A. B., and Nesbett, F. B , *J. Biol. Chem* , **150,** 413 (1943).
10. Boxer, G E , and Stetten, D , Jr , *J Biol Chem* , **153,** 617 (1944)
11. Levin, L., *Science*, **96,** 477 (1942).

SOURCES OF ACETIC ACID IN THE ANIMAL BODY*

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The formation of acetic acid as a product of intermediary metabolism has frequently been postulated. Animal tissues can metabolize acetic acid at a rapid rate (1); so that its concentration in the body fluids at any given time may be too small to permit its detection by means of the analytical methods available. A demonstration of acetate formation *in vivo* will be feasible, however, if acetate enters into intermediary reactions leading to a fixation of the C_2 unit in compounds which are either stored in the tissues or excreted. Three reactions are known to occur in intact animals which involve utilization of the carbon atoms of acetic acid; *viz.*, acetylation of foreign amines (2), formation of ketone bodies (3), and formation of cholesterol (4). The appearance of isotope in the product of any one of these processes will, after administration of an isotopic compound, be taken as evidence for the formation of acetic acid from the compound tested. Of the three processes, the acetylation of foreign amines appears to be the most suitable one for demonstration of acetate formation, for the quantities of ketone bodies excreted by normal animals are insufficient for isolation, while cholesterol synthesis proceeds at a rate so slow that extended feeding periods are required in order to demonstrate utilization of acetate.

Proof for acetylation of amines *in vivo* by acetic acid was first furnished by Bernhard (2) who observed the excretion of deuterio acetyl derivatives when a foreign amine and deuterio acetic acid were administered to dogs. The same investigator employed the acetylation reaction to demonstrate the biological oxidation of ethanol to acetic acid by the intact animal (5).

We have studied the acetylation reaction by acetic acid in somewhat greater detail.[1] The quantitative results suggested that acetate was produced by animal tissues in relatively large amounts and raised the question as to its origin. The findings reported here are interpreted on the assumption that acetic acid is a direct acetylating agent and that the appearance of deuterio acetyl groups is proof of the intermediary formation of deuterio acetic acid from the isotopic test substance.

The formation of acetic acid as an oxidation product of fat is suggested in the β oxidation scheme of Knoop, although the exact nature of the C_2 units arising in the breakdown of fatty acids has never been determined.

[1] Unpublished results.

We have prepared a number of deuterium-containing compounds and have tested their ability to give rise to isotopic acetyl groups. The findings obtained are entirely consistent with the β oxidation theory.

The question whether acetic acid is formed as an intermediate in the oxidative breakdown of carbohydrates is still unanswered. The oxidative decarboxylation of pyruvic acid known to be carried out by microorganisms (6) has yet to be demonstrated in intact animal tissues. The dismutation of pyruvate into lactate and acetate, demonstrated by Krebs in liver slices (7), may involve a different mechanism. When deuterio alanine was administered to rats, high concentrations of deuterium appeared in the acetyl group of the acetylphenylaminobutyric acid excreted. This finding leads to the conclusion that the oxidative decarboxylation of pyruvate to acetyl occurs in intact animal tissues.

<center>EXPERIMENTAL</center>

<center>*Preparation of Isotopic Compounds*</center>

Sodium Deuterio Acetate—This compound was prepared from malonic acid as previously described (4). It contained 27.6 atom per cent excess deuterium.

Dideuterio Succinic Acid—This compound was prepared from fumaric acid as described before (4). It contained 26.9 atom per cent excess deuterium.

Sodium Dideuterio Propionate—This compound was prepared as previously described (8). It contained 34.4 atom per cent excess deuterium.

Sodium Dideuterio Butyrate—The sodium salts of the α,β- and β,γ-dideuterio butyric acids were prepared as previously described (4). They contained 22.0 and 16.0 atom per cent excess deuterium respectively.

Sodium n-Deuterio Valeric Acid—This compound was prepared by the platinum-catalyzed exchange reaction between sodium valerate and D_2O. It contained 17.3 atom per cent excess deuterium.

Deuterio Ethyl Palmitate—Dry normal acetylene was circulated in a closed system through a hydrating mixture of 9 cc. of D_2O, 1.2 cc. of concentrated H_2SO_4, and 1.0 gm. of $HgSO_4$ kept at 70°. After most of the water in the exit gases was removed by a reflux condenser, the acetaldehyde was condensed in a dry ice trap. Fresh acetylene was continuously added to replace that reduced to acetaldehyde or dissolved in the liquid acetaldehyde. After 10 liters of acetylene had been introduced, the uptake of acetylene ceased. The contents of the dry ice trap were brought to 0°, when much gas, presumably dissolved acetylene, was evolved.

The acetaldehyde was reduced with hydrogen over a nickel catalyst (9). Hydrogen saturated with deuterio acetaldehyde at −30° was slowly passed

over this catalyst kept at 150°.   The exit gases were passed through a trap
kept at −10°.

The liquid condensed at −10° was warmed to 25°.   A small amount of
unchanged acetaldehyde evaporated.   About 10 cc. of liquid (alcohol +
water) were obtained.   The alcohol in this was converted into deuterio
ethyl 3,5-dinitrobenzoate, 33.0 gm., m.p. 93°, 16.5 gm. of which were
hydrolyzed by boiling alkali.   The resulting alcohol (3.2 cc.) was dried
with K_2CO_3 and warmed on a steam bath for 3 hours with 26 gm. of palmi-
toyl chloride.   The reaction mixture was diluted with ether, washed with
K_2CO_3 solution, and with water, and distilled.   The main fraction, 12.26
gm., b.p. 140–150° (2 mm.), contained 3.40 atom per cent excess deuterium,
from which the methyl group of the ethyl alcohol can be calculated to
contain 40.8 atom per cent excess deuterium.

In order to determine the position of the deuterium in the deuterio
alcohol another sample of acetaldehyde was reduced to alcohol and con-
verted to ethyl butyrate, b.p. 119.5–121.5°.   This contained 13.1 atom per
cent excess D, corresponding to ethyl alcohol with 26.2 atom per cent
deuterium.   A sample (1.96 gm.) was hydrolyzed with alkali and the alco-
hol oxidized with $KMnO_4$ in dilute H_2SO_4.   The acetic acid was distilled
off and converted into silver acetate (1.4 gm.), of which 903 mg., burned
for the deuterium analysis, yielded 582 mg. of silver (theory 583 mg.) and
contained 50.3 atom per cent excess deuterium.   If all the deuterium in
the ethyl alcohol were in the methyl group, the derived silver acetate should
contain 52.4 atom per cent excess.   The agreement is excellent; very little
deuterium can have been lost by exchange during the conversion of ethyl
alcohol to acetic acid (10), and little can have entered the acetylene by
exchange with the hydrating mixture (11).

10,11-Dideuterio Ethyl Undecylate—Ethyl undecylenate was hydrogen-
ated in ether solution with D_2 gas.   The ester contained 7.19 atom per cent
excess D, corresponding to an isotope concentration of 31.2 atom per cent
excess in the terminal methyl group.

Deuterio Alanine—5 cc. of freshly distilled pyruvic acid (0.072 mole)
and 8 cc. of concentrated NH_3 solution (0.144 mole) were dissolved in
100 cc. of 80 per cent ethyl alcohol and reduced with D_2 gas and palladium
black (12).   After 5 hours, 1600 cc. of gas were absorbed.   The alanine
isolated contained 10.0 atom per cent excess deuterium.   N (Kjeldahl)
found, 15.5; calculated for $C_3H_{6.3}D_{0.7}O_2N$, 15.6 per cent.

0.1000 gm. of deuterio alanine was mixed with 1.7002 gm. of normal
alanine, dissolved in 300 cc. of H_2O, and the pH brought to 5.5 by the
addition of phosphoric acid.   1 mole of chloramine-T was added and the
solution kept at 40° for 1 hour.   The acetaldehyde was distilled off.   To
the distillate (100 cc.) were added 20 cc. of 85 per cent H_3PO_4 and 1.5 gm.

of $KMnO_4$. The mixture was kept at room temperature for 18 hours, then refluxed for 15 minutes. Half the water was distilled over, the excess $KMnO_4$ was reduced with H_2O_2, and the bulk of the acetic acid was distilled with four successive additions of 50 cc. of water. The distillate was neutralized with K_2CO_3 and taken to dryness. The potassium acetate, extracted with hot absolute alcohol and dried at 100° *in vacuo*, contained 18.2 atom per cent excess deuterium.

From the isotope concentration of the alanine and the acetate derived from it, it can be calculated that the α-hydrogen atom of the alanine contained 15.4 and the methyl group 18.2 atom per cent excess deuterium.

Ethyl Deuterio Myristate—Deuterio myristic acid with 29.8 atom per cent excess D, prepared by catalytic exchange with D_2O, was esterified with ethyl alcohol.

Ethyl Deuterio Stearic Acid—40 cc. of linseed oil were dissolved in 70 cc. of dry isoamyl ether and reduced catalytically with D_2 and Pt. The product was hydrolyzed with 7 per cent alcoholic KOH, and the free fatty acids were esterified with ethyl alcohol. The mixture of esters, b.p. 162–177° (2 mm), weighed 27.4 gm. and contained 5.80 atom per cent excess D.

Feeding Experiments—Rats weighing about 250 gm. were used in all experiments. The test substance was incorporated into the stock diet of the following composition: 71 per cent corn-starch, 16 per cent casein, 5 per cent yeast, 4 per cent salt mixture (13), 2 per cent cod liver oil, and 2 per cent sodium chloride. The large daily consumption of water resulting from the high content of sodium chloride in the diet kept the isotope concentration of the body fluids low. In the experiment in which ethyl deuterio myristate was fed, the diet consisted of casein 85 per cent, yeast 5 per cent, bone meal 2 per cent, cod liver oil 2 per cent, salt mixture 4 per cent (13), and sodium chloride 2 per cent. The animals consumed from 10 to 15 gm. of these diets daily. In all experiments *dl*-phenylaminobutyric acid, prepared according to du Vigneaud and Irish (14), was incorporated into the stock diets. The animals received 100 mg. per day of this substance per 100 gm. of body weight.

Isolation of l-Acetylphenylaminobutyric Acid—The pooled urines were made alkaline to phenolphthalein and extracted continuously with ether for 5 hours. The urines were then acidified to Congo red and again extracted with ether for 24 hours. The ether extract from the acidified urine was concentrated and the residue was taken up in a small volume of hot water. The acetyl derivative which crystallized required, as a rule, only one recrystallization from hot water with the addition of charcoal to yield a product melting at 179–180°. The purity was checked by determination of nitrogen content. Calculated 6.33 per cent; found in all samples, from 6.2 to 6.4 per cent. In some cases the optical rotation of the excreted

acetyl derivative was determined and was found to correspond to that of the pure l isomer (15).

Isolation of Fatty Acids after Feeding Ethyl Deuterio Myristate—At the end of the 3 day feeding period the animals were killed, the entire gastro-

TABLE I

Formation of Deuterio Acetyl Groups from Deuterio Compounds

Compound fed			Duration of experiment	Body water	Acetyl group of acetyl-phenyl-amino-butyric acid	Coefficient of utilization		Cholesterol
Experiment No						Total*	Specific†	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	mM per 100 gm body weight	*atom per cent excess D*	*days*	*atom per cent excess D*	*atom per cent excess D*			*atom per cent excess D*
1 Sodium deuterio acetate	1.55	27.6	8	0.07	2.00	15.6	15.6	0 200
2 Sodium dideuterio propionate	0 94	34.4	5	0.04	0.08	0.5		0.00
3 Dideuterio succinic acid	1.60	26.9	3	0.03	0.16	0 6		
4 Sodium α,β-dideuterio butyrate	1.60	22 0	8	0.05	0 47	1.9	3 8 (α)	0 05
5 Sodium β,γ-dideuterio butyrate	1.75	16 0	8	0.04	0 74	3.8	7.6 (γ)	0 12
6. Sodium n-deuterio valerate	1 60	17.3	8	0 04	0 22	0.9	4 0 (α)	0 06
7 Ethyl 10,11-dideuterio undecylic acid	1.60	7 2	8	0 07	0.08	0 3	0.5 (ω)	
8 Ethyl deuterio myristate	1.13	26 0	4		0.56	0.6	5 5 "	
9 Ethyl 9,10-dideuterio stearate	1.60	5.8	4	0 02	0.07	0.2		
10 Deuterio ethyl palmitate	1.6	3 4	8	0 06	2 8	14.4	14.4	0 16
11 Deuterio dl-alanine	1.2	10 0	4	0.03	0.64	7.6	9.8 (β)	

* Calculated for all deuterium atoms in the molecule.

† Calculated for the deuterium attached to the carbon atoms designated by the Greek letter.

intestinal tract removed, and the livers and the remainder of the carcasses hydrolyzed with ethanolic KOH. The carcasses yielded 21.1 gm. of fatty acids containing 0.57 atom per cent, and the livers yielded 0.383 gm. of fatty acids containing 4.10 atom per cent excess deuterium.

Isolation of Cholesterol—Cholesterol was isolated from the hydrolyzed

rat carcasses and purified via the dibromide. The isotope concentrations are given in Column 6 of Table I.

In Table I are listed the isotope concentrations in the acetyl group of acetylphenylaminobutyric acid excreted in the urine of rats to the diet of which *dl*-phenylaminobutyric acid and the deuterium-containing test substance had been added. We have in all cases calculated, on the assumption that the deuterium is present only in the acetyl group, the isotope concentration of the hydrogen in the acetyl group by multiplying by 5 the deuterium concentration found in the isolated acetylphenylaminobutyric acid $(C_{12}H_{15}NO_3)$. This assumption is not strictly valid, for in our experiments some deuterium is present in the body fluids as a result of the oxidation of the deuterio compounds fed, and it is known that deuterium may enter the α position as well as the acetyl group from the body fluids (15, 16). A small fraction of the deuterium found in the excreted acetylphenylamino-butyric acid may, therefore, arise from this source. The deuterium concentration of the excreted acetylphenylaminobutyric acid due to these reactions cannot be greater than 4/15 that of the body fluids. The isotope concentration of the hydrogen of the acetyl group of this compound must, therefore, be at least 5 times higher, 20/15 or 4/3 that of the body fluids, before utilization of the organic compound for acetylation can be regarded as proved.

As the amount of deuterium administered to the animals in the test substance depends not only on the number of moles fed and the isotope concentration of the hydrogen atoms but also on the number of hydrogen atoms per mole of compound, we shall define a quantity in Equation 1 called the coefficient of utilization of deuterium for acetate formation.

$$(1) \qquad\qquad \text{Coefficient of utilization} = \frac{1000\ D_{ac}}{D_f.M.n_H}$$

where

D_{ac} = isotope concentration of the excreted acetyl group
D_f = "   "   in " compound fed
M = mM of test substance fed per 100 gm. body weight
n_H = number of hydrogen atoms per mole of test substance

The denominator of Equation 1 is the number of milliequivalents of deuterium administered in the test compound. The acetylating agent itself should have the highest coefficiency of utilization. The numerical value for acetate is 15.6. A coefficient of utilization greater than unity may be taken to signify direct utilization of the test substance. Smaller values may be the result of incorporation of deuterium of the body fluids

into the acetyl precursor or phenylaminobutyric acid. The coefficient of utilization may be calculated not only for all the hydrogen atoms in the test compound but also for hydrogen atoms attached to individual carbon atoms. In the latter case, D_f is the isotope concentration of the hydrogen at the specific carbon atom and n_H is the number of such hydrogen atoms. All other terms have the same significance. In case only some of the hydrogen atoms are utilized for acetyl formation, the specific coefficient of utilization will be of greater significance than that calculated for the whole molecule.

The observation that none of the test substances gave rise to acetyl groups with isotope concentrations exceeding those resulting from acetate itself, or had a higher coefficient of utilization, lends strong support to the contention that acetic acid (or a functional derivative) is the chief acetylating agent. As acetate is utilized for the synthesis of cholesterol, deuterio cholesterol should be formed whenever deuterio acetyl derivatives appear. This proved to be the case throughout. Up to the present time strict correlation between formation of deuterio acetyl and synthesis of deuterio cholesterol has been observed. The specific function of acetate to act as a precursor of cholesterol is emphasized by this relationship.

Ethyl Alcohol $(CH^*_3CH_2OH)$[2]—According to Bernhard (5), ethanol seemed, on the basis of isotope concentrations in the excreted acetyl group, to be a more efficient acetylating agent than acetic acid. This result could be taken to mean that the actual acetylating agent is not acetic acid but is a C_2 unit which is produced from ethanol more readily than from acetate. In our experiments deuterio ethanol, administered as ethyl palmitate, was as effective a source of acetyl as deuterio acetate; the two compounds have nearly the same coefficient of utilization. Though this finding does not eliminate the possibility that the effect of ethanol is due to an acetylation mechanism different from that involving acetate, the quantitative agreement in the behavior of ethanol and of acetate suggests strongly that ethanol acts as an acetylating agent by way of acetic acid.

Succinic Acid $(COOHCH^*_2CH^*_2COOH)$—Although the deuterium concentration in the acetyl group excreted after the ingestion of succinate is more than 4 times as high as that in the body fluid, the coefficient of utilization is less than unity. This result suggests that a small fraction of the succinate may be converted to acetate. The occurrence of such a reaction in bacteria has been demonstrated by Slade and Werkman (17).

Propionic Acid $(CH^*_3CH^*_2COOH)$ *and Alanine* $(CH^*_3CH^*NH_2COOH)$—When deuterio propionate was administered, the acetyl derivative excreted contained no significant excess of deuterium (Table I, Experiment 2). The failure of deuterio propionic acid to form deuterio cholesterol has

[2] In all cases the deuterium atoms are in the positions labeled by an asterisk..

already been reported (4). On the other hand, alanine produces isotopic acetyl groups (Table I, Experiment 11). Since the oxidative deamination of alanine to pyruvic acid occurs readily in the animal body, pyruvic acid and alanine can be considered as equivalent with respect to the acetylation reaction.

No decision can be made, on the basis of these results alone, as to the sequence of steps involved in the pyruvate-acetyl transformation. Pyruvic acid may, as proposed by Knoop (18), act as an acetylating agent by condensation with an imino acid and subsequent decarboxylation of the condensation product. Or, if oxidative decarboxylation to acetic acid is the primary step, then acetic acid will be the acetylating agent proper. Regardless of the mechanism by which pyruvic acid supplies acetyl groups, the present findings establish the oxidative breakdown of alanine (and pyruvic acid) to acetyl in the intact animal. The coefficient of utilization for alanine, with reference to the methyl group only, has been calculated to be 9.8, or two-thirds of that for acetate. This figure indicates that at least two-thirds of the dietary alanine must have been degraded to acetyl. Even if alanine or pyruvate were converted quantitatively to acetyl, identical values could not be expected, as some of the deuterio methyl groups of dietary alanine must be stored in the tissue proteins and are, therefore, not available. The magnitude of the coefficient of utilization suggests that the loss of deuterium due to enolization of the pyruvic acid derived from alanine was slow. The failure of deuterio propionate, which is a glycogenic compound, to yield isotopic acetyl indicates that the primary step in its metabolism is not α oxidation to pyruvic acid. Oxidation at the β-carbon atom appears to be the only alternative.

Butyric Acid—The deuterium concentrations observed (4) in cholesterol after feeding the two butyric acids containing deuterium on the α,β and β,γ positions respectively were low but significant. The analytical values were interpreted as indicating a partial conversion of butyrate to acetate rather than direct utilization of butyrate for cholesterol synthesis. This is substantiated by the present finding that α,β- as well as β,γ-dideuterio butyric acid forms deuterio acetyl groups. It is clear that a mechanism exists by which butyric acid is oxidized to 2 moles of acetate. If acetoacetic acid represents the primary oxidation product, then, as shown by

$$CH_2D \cdot CHD \cdot CH_2COOH \qquad\qquad CH_3CHD \cdot CHD \cdot COOH$$
$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$
$$CH_2D \cdot CO \cdot CH_2COOH \qquad\qquad CH_3 \cdot CO \cdot CHC \cdot COOH$$
$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$
$$CH_2D \cdot COOH + CH_3COOH \qquad\qquad CH_3COOH + CH_2DCOOH$$
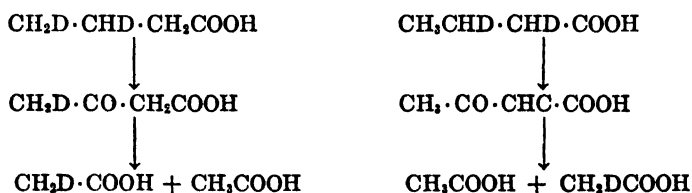
the accompanying schemes, the isotopic label on the β-carbon atom will be removed from both acids. The hydrogen atoms at the α-carbon atom of β-keto acids are known to enolize readily and loss of deuterium will occur with acetoacetate derived from the α,β-dideuterio butyric acid. However, this loss appears not to be complete before the breakdown of labile aceto-acetate into stable acetate has occurred. The hydrogen at the γ-carbon atom is less readily enolized, and most of the isotope will be retained. In accord with these considerations, more than twice as high a coefficient of utilization is found for the β,γ-dideuterio butyrate as for the α,β acid. Similar results on the relative lability of the hydrogen atoms in the α and γ positions have been reported by Morehouse (19), who found that β-hydroxybutyrate containing deuterium was excreted only after adminis-tration of β,γ-dideuterio butyrate.

The fact that both α,β- and β,γ-deuterio butyric acids give rise to deuterio acetyl groups proves that butyric acid can break down to 2 mole-cules of acetic acid.

Since oxidation to acetoacetic acid will in both acids result in the loss of the deuterium at the β-carbon atom, the coefficients of utilization of the other deuterium atoms at the α or γ positions will be more significant than that calculated for the whole molecule. The value found for the γ-deute-rium shows that not less than half of the butyrate was metabolized to acetic acid by way of acetoacetic acid.

In the present experiment, after the ingestion of β,γ-dideuterio butyric acid, the deuterium concentration in the cholesterol was 3 times that in the body fluids (Table I, Experiment 5). From an earlier experiment on deuterio cholesterol formation in which β,γ-dideuterio butyrate was found to be considerably less effective than deuterio acetate, we concluded (4) that acetate was not the main product of butyrate metabolism. The quantitative difference between the two findings may in part be due to the use, in the previous experiments, of a high protein diet, in contrast to the high carbohydrate diet in the work here reported. The present estimation of butyrate utilization appears to be more precise, for acetyl production can be measured more accurately by the acetylation technique.

The difference in stability of hydrogen atoms at the α and γ positions respectively is reflected by the appreciably higher isotope content in cholesterol after the administration of β,γ-dideuterio butyrate, in contrast to α,β-dideuterio butyrate.

Higher Fatty Acids—Only a limited number of isotopic fatty acids have as yet been tested as to their ability to yield deuterio acetyl groups. Of these acids only myristic acid, which had been prepared by catalytic ex-change (20) and presumably contained the isotope uniformly distributed over the carbon chain, formed acetyl groups with a significant deuterium

concentration. If, as in Knoop's scheme of β oxidation, β-keto acids are formed from which C_2 units are successively removed, then the deuterium should be lost entirely at the β position and rapidly exchanged at the α-carbon atom exactly as in the case of acetoacetate derived from α,β-dideuterio butyrate. When the fatty acid chain has been shortened to the 4-carbon stage, the γ-carbon atom which has been the ω-carbon atom in the original acid, will retain most of its isotope, and the acetic acid derived from the 2 carbon atoms furthest from the carboxyl group of the fatty acid will be the richest in isotope. The maximum deuterium concentration to be expected in acetate formed by the breakdown of higher fatty acids will then be of a magnitude similar to that given by β,γ-dideuterio butyrate. As the deuterium concentration found in the acetyl group following administration of isotopic myristic acid was roughly equal to that obtained after α,β-dideuterio butyrate feeding, the coefficient of utilization for the total molecule is small. On the other hand, calculation for the terminal methyl group yields a value of 5.5, intermediate between those calculated for the α- and the γ-hydrogen atoms of butyric acid. However, as the higher fatty acids are partly stored in the fat depots, whereas lower ones, such as butyric acid, are immediately metabolized, not all of the long chain fatty acids administered will be immediately available as a source of acetate.

From the isotope concentration and total amount of the fat of the animal it can be calculated that 10 per cent of the deuterium in the myristic acid was deposited. When palmitic acid had been fed (21), 44 per cent was found in the depots. After the administration of 9,10-deuterio stearic acid, the excreted acetyl groups contained less than significant amounts of isotope. In this experiment, the deposition of the labeled fatty acid must have been considerable (22).

Of the two odd numbered fatty acids tested, n-valeric acid, containing deuterium evenly distributed along the carbon chain, showed a low but significant effect. If oxidation at the β-carbon atom is again the initial step in the breakdown, then 1 molecule of propionic acid and 1 molecule of acetic acid may be formed. The acetic acid will have retained only the small amount of α-deuterium which was not exchanged in the β-keto acid stage, and no acetic acid is formed from propionic acid. On this basis the coefficient of utilization for the deuterium atoms at the α-carbon atom is found to be 4.0. This is in close agreement with the value found for the α-deuterium atoms of butyric acid. n-Valeric acid has been reported to form ketone bodies in diabetic animals (23). The β oxidation scheme would again account for all the experimental findings concerning n-valeric acid if the acetoacetic acid formed in ketosis is a secondary product arising from the condensation of acetic acid.

After the ingestion of 10,11-dideuterio undecylic acid, acetyl containing

only normal hydrogen was found. Successive β oxidations of this compound would yield 4 molecules of non-isotopic acetic acid, but α,β-dideuterio propionic acid derived from carbon atoms 9, 10, and 11 would not be converted to acetate.

All of our present findings are compatible with the concept of β oxidation and the successive removal of C_2 units in the form of acetic acid. None of our observations for odd numbered, as well as for even numbered, fatty acids indicates initial oxidation at the α-carbon atom.

The conclusions drawn from the experiments presented here presumably are valid only for the organs in which the acetylation reaction and cholesterol formation occur. There is adequate evidence that the liver is the site of acetylation, which is not known to be the case of cholesterol. The statement that alanine or pyruvate is oxidized to acetyl may hold true only for the liver, which is known (24) to be the site of acetylation. It is impossible to decide whether the pyruvate (or phosphopyruvate) formed in other tissues follows the same metabolic pathway as the pyruvate resulting from oxidative deamination of alanine in the liver.

SUMMARY

1. The observation of Bernhard that oral administration of deuterio acetate with an amino acid foreign to the organism leads to excretion of an isotopic acetyl derivative has been confirmed. Advantage has been taken of this effect to demonstrate formation of acetic acid in the normal rat.

2. Deuterium-containing test substances were added to the stock diet of normal rats in conjunction with phenylaminobutyric acid, and the isotopic concentration of the excreted acetyl derivative was determined.

3. Deuterio acetyl groups were excreted after administration of labeled ethanol, butyric acid, alanine, n-valeric acid, and myristic acid, but not from propionic acid and 10,11-dideuterio undecylic acid.

4. A considerable fraction of butyric acid is metabolized to yield 2 moles of acetic acid.

5. Only those compounds which gave rise to deuterio acetyl groups formed deuterio cholesterol; this finding supports the view that acetate is a specific cholesterol precursor.

6. The result obtained with deuterio alanine is compatible with the oxidative decarboxylation of pyruvate to acetyl in the intact rat.

7. The data are in agreement with Knoop's concept of β oxidation in its application to odd as well as to even numbered fatty acids.

BIBLIOGRAPHY

1. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem*, **150**, 413 (1943).
2. Bernhard, K., *Z. physiol. Chem*, **267**, 91 (1940)
3. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).

4. Bloch, K., and Rittenberg, D., *J Biol. Chem.*, **145**, 625 (1942).
5. Bernhard, K., *Z. physiol. Chem.*, **267**, 99 (1940).
6. Neuberg, C , and Karczag, L , *Biochem. Z.*, **36**, 76 (1911).
7. Krebs, H. A , and Johnson, W A., *Biochem J.*, **31**, 640 (1937)
8. Rittenberg, D., Schoenheimer, R , and Evans, E. A., Jr , *J. Biol. Chem.*, **120**, 503 (1937).
9. Sabatier, P., and Senderens, J. B., *Compt. rend. Acad.*, **137**, 301 (1903).
10. Bok, C. L D , and Geib, K. H., *Z. phys. Chem.*, *Abt. A*, **183**, 353 (1939)
11. Reyerson, L H , and Yuster, S., *J. Am. Chem. Soc* , **56**, 1426 (1934).
12. Knoop, F., and Oesterlin, H , *Z. physiol Chem.*, **170**, 186 (1927).
13. Osborne, T. B., and Mendel, L B., *J. Biol. Chem* , **15**, 311 (1913).
14 du Vigneaud, V , and Irish, O T., *J Biol. Chem.*, **122**, 349 (1937–38).
15. du Vigneaud, V., Cohn, M , Brown, G B., Irish, O T., Schoenheimer, R., and Rittenberg, D , *J. Biol Chem* , **131**, 273 (1939)
16 Fishman, W. H , and Cohn, M , *J. Biol. Chem* , **148**, 619 (1943).
17. Slade, H. D , and Werkman, C H , *Arch Biochem* , **2**, 97 (1943)
18. Knoop, F , *Beitr chem Physiol. u Path* , **6**, 150 (1905).
19 Morehouse, M G , *J Biol Chem* , **129**, 769 (1939).
20. van Heyningen, W. E , Rittenberg, D , and Schoenheimer, R , *J Biol. Chem.*, **125**, 495 (1938)
21 Stetten, D., Jr , and Schoenheimer, R , *J Biol Chem* , **133**, 329 (1940).
22 Schoenheimer, R , and Rittenberg, D., *J Biol Chem* , **120**, 155 (1937).
23 MacKay, E M , Wick, A N , and Barnum, C P , *J Biol. Chem* , **136**, 503 (1940).
24 Klein, J R , and Harris, J S , *J Biol. Chem* , **124**, 613 (1938)

SOME ASPECTS OF THE METABOLISM OF LEUCINE AND VALINE*

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Cholesterol, which is formed by biological synthesis, contains angular methyl groups and branched side chains. A few other tissue constituents exist which contain branched chains, notably valine and leucine, but these are all essential dietary constituents. It has been shown (1) that acetic acid participates in the synthesis of the entire structure of cholesterol, but no obvious mechanism is known by which acetic acid could react to form branched carbon chains. Our attempts to find specific cholesterol precursors other than acetic acid have been unsuccessful; in all instances in which the administration of a labeled test substance led to the formation of deuterio cholesterol, the effect has been attributable to the intermediary formation of deuterio acetic acid (2).

Experiments were carried out to test the effect of deuterio valine and deuterio leucine on the biological formation of cholesterol. Cholesterol isolated from animals which had received deuterio leucine contained significant concentrations of isotope, but only normal cholesterol was formed when deuterio valine was the test substance. Of the two branched chain fatty acids which are likely to be degradation products of leucine and valine respectively, deuterio isovaleric acid gave an effect similar to that of leucine, whereas deuterio isobutyric acid, like valine, failed to produce isotopic cholesterol.

The appearance of deuterium in cholesterol following administration of deuterio leucine is evidence of the ability of leucine to supply carbon atoms for sterol synthesis, but provides no proof of a specific utilization of the isopropyl groups. Leucine is a ketogenic substance; *i.e.*, a source of acetoacetic acid. Though it is recognized that in ketosis the acetone bodies can arise either as primary oxidation products or by recondensation of acetic acid, little is known as to the metabolism of acetoacetate or other ketogenic substances under normal conditions. In the preceding paper evidence is presented that extensive hydrolysis of acetoacetate to acetate can occur in normal animals. Intermediary formation of acetic acid may be detected by the appearance of deuterio acetyl groups in acetylamino acids. This

method has now been employed to investigate the degradation of leucine, valine, isovaleric acid, and isobutyric acid. The acetyl groups of the excreted acetylphenylaminobutyric acid contained deuterium when appropriately labeled leucine or isovaleric acid was fed, but contained no excess of isotope after administration of deuterio valine or deuterio isobutyric acid. Thus, only the two substances which are known to be ketogenic produce acetic acid. Since deuterio cholesterol is formed from the same two substances, their effect in cholesterol synthesis may well be the result of intermediary formation of acetic acid, which in turn is utilized for cholesterol synthesis.

Acetic acid supplies carbon atoms for the nucleus as well as for the side chain of the cholesterol molecule (1). The cholesterol isolated after deuterio leucine feeding was degraded into the isooctane-isooctene mixture corresponding to the cholesterol side chain and the polynuclear hydrocarbon $C_{19}H_{30}$. The isotope concentrations in the two fragments were almost equal, as was the case with cholesterol resulting from deuterio acetate feeding. This finding strengthens the view that a similar mechanism is responsible for the appearance of deuterium in cholesterol, and that leucine is probably not a specific cholesterol precursor.

EXPERIMENTAL

Preparation of Deuterio dl-Leucine. Isocaproic Acid—Since pure isocaproic acid can be separated only with difficulty from commercial mixtures of the isomeric acids, it was prepared by a modification of a method described by Braun (3). Isobutyraldehyde[1] was condensed with malonic acid and the resulting isopropylacrylic was hydrogenated. To 136 ml. of freshly distilled isobutyraldehyde were added 104 gm. (1 mole) of powdered malonic acid and 100 ml. of dry pyridine. The mixture was kept at room temperature for 12 hours, and was then heated under a reflux for 6 hours. The excess aldehyde was distilled off and the mixture refluxed again for 1 hour. The cooled reaction mixture was diluted with 3 volumes of water, made acid to Congo red with sulfuric acid, and extracted with ether. The solvent was removed from the dried ether solution and the residue was distilled *in vacuo*. The fraction distilling at 112–114° at 20 mm. of Hg was collected. 53 gm. of isopropylacrylic acid were obtained (47 per cent of theory). 35 gm. of the unsaturated acid were dissolved in 350 ml. of absolute ethanol and hydrogenated in the presence of active platinum. Distillation of the reduction product yielded 32 gm. of isocaproic acid, b.p. 103–104° at 13 mm. of Hg.

Deuterio Isocaproic Acid—The isocaproic acid was converted into the sodium salt and exchanged with D_2O in the presence of activated platinum

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[1] We are indebted to Dr. Roland Kapp of the National Oil Products Company for supplying us with the isobutyraldehyde

in a sealed flask. The contents were shaken at 130° for 14 days. The isocaproic acid recovered contained 47.9 atom per cent excess of carbon-bound deuterium.

Deuterio *dl*-leucine was prepared by bromination of the isotopic isocaproic acid and treatment of the α-bromoisocaproic acid with ammonia (4). An over-all yield of 53 per cent, calculated for isocaproic acid, was obtained. N, Kjeldahl, found, 10.5 per cent; calculated for $C_6H_{8.2}D_{4.8}NO_2$, 10.3 per cent. The compound contained 37.0 atom per cent excess deuterium. Of the 13 hydrogen atoms of leucine, the 2 of the amino group and that of the carboxyl group must be non-isotopic. The remaining 10

TABLE I

Formation of Deuterio Acetyl Groups and Deuterio Cholesterol from Deuterio Compounds

Compound fed			Duration of experiment	Body water	Acetyl group of acetyl-phenyl-amino-butyric acid	Coef-ficient* of utilization	Cho-lesterol
	mμ per 100 gm body weight	*atom per cent excess D*	*days*	*atom per cent excess D*	*atom per cent excess D*		*atom per cent excess D*
Sodium deuterio acetate	1.55	27.6	8	0.07	2.00	15.6	0.20
Deuterio *dl*-leucine	1.13	48.1	16	0.15	2.50	5.1	0.56
Sodium deuterio iso-valerate	1.10	23.5	8	0.10	1.19	5.1	0.18
Deuterio *dl*-valine	1.10	34.0	8	0.04	0.08	0.3	0.03
Sodium deuterio iso-butyrate	1.10	24.0	8	0.03	0.07	0.4	0.03

* Coefficient of utilization $= D_{ac}/n_H \times mM \times D_f$, where $D_{ac} =$ per cent deuterium in the acetyl group, $n_H =$ total number of hydrogen atoms in the compound fed, $D_f =$ per cent deuterium in the compound fed.

hydrogen atoms will, therefore, contain $37.0 \times 13/10 = 48.1$ atom per cent excess deuterium. As this value is almost identical with that found for the carbon-bound hydrogen of isocaproic acid, the hydrogen lost from the α-carbon atom of isocaproic acid by bromination must have had the same isotope concentration as the remainder of the carbon-bound hydrogen atoms. The catalytic exchange of isocaproic acid must, therefore, have led to fairly uniform distribution of deuterium along the carbon chain.

In Table I, the isotope concentration of *dl*-leucine given is not that actually found, *i.e.* 37 per cent, but 48.1 per cent, *i.e.* the deuterium concentration of the 10 carbon-bound hydrogen atoms. This is justified because in acetate formation only the carbon-bound hydrogen atoms need be considered.

Deuterio dl-Valine. Isovaleric Acid—The compound was prepared from isopropyl bromide and diethyl malonate according to Marvel and du Vigneaud (5). For introduction of deuterium, sodium isovalerate was exchanged with D_2O in the presence of activated platinum. The reaction mixture was shaken at 130° in a sealed flask for 12 days. The isovaleric acid recovered contained 34.8 atom per cent excess deuterium. The deuterio isovaleric acid was brominated and the bromo acid allowed to react with ammonia (6). N, Kjeldahl, found, 11.7 per cent; calculated for $C_5H_{7\ 3}D_{3\ 7}NO_2$, 11.7 per cent. The amino acid contained 24.7 atom per cent excess deuterium. As in the case of deuterio leucine, the 3 hydrogen atoms in the amino group and the carboxyl group must be non-isotopic. The carbon-bound hydrogen atoms will, therefore, contain $24.7 \times 11/8 = 34.0$ atom per cent excess deuterium. This compares with a deuterium concentration of 34.8 per cent found for isovalerate; the rate of exchange must, therefore, have been roughly the same for all carbon-bound hydrogen atoms of isovaleric acid. The isotope concentration for valine given in Table I is again that of the carbon-bound hydrogen atoms; *i.e.*, 34.0 atom per cent.

Deuterio Isovalerate and Deuterio Isobutyrate—The isotopic isovaleric acid employed in the feeding experiment was prepared in the same manner as that used for valine synthesis. It was fed in the form of the sodium salt which contained 23.5 atom per cent excess deuterium. Isotopic isobutyrate was prepared from commercial isobutyric acid by exchange of the sodium salt with D_2O in the presence of activated platinum. The mixture was shaken at 127° for 16 days and the sodium isobutyrate recovered contained 24.0 atom per cent excess deuterium.

Feeding of dl-Deuterio Leucine to Mice—Twenty adult mice were placed in three groups on a stock diet containing 77 per cent starch, 6 per cent casein, 5 per cent yeast, 6 per cent Wesson oil, 2 per cent cod liver oil, 2 per cent salt mixture (7). Each animal consumed about 3 gm. daily of this diet. In addition, each mouse received per day 40 mg. of *dl*-leucine containing 37.0 atom per cent excess deuterium and 75 mg. of sodium chloride, mixed with the stock diet. The sodium chloride was added in order to increase consumption of drinking water and thereby maintain the deuterium concentration of the body fluids at a low level. After 8 days ten mice of Group I were killed. A sample of body water was distilled from the tissues and cholesterol isolated from the pooled carcasses in the usual fashion. The body water contained 0.055 and the cholesterol 0.136 atom per cent excess deuterium.

The four mice of Group II were kept on the deuterio leucine-containing diet for 15 days, when they were killed. The body water contained 0.061 atom per cent excess deuterium. Cholesterol was isolated from the pooled animal carcasses as the digitonide. The cholesterol regenerated from the digitonide contained 0.232 atom per cent excess deuterium.

The remaining six animals (Group III) were killed after they had received the deuterio leucine-containing stock diet for a period of 22 days. The body water of the pooled tissues contained 0.049 per cent D_2. Cholesterol from the combined carcasses was isolated as the digitonide. The cholesterol digitonide contained 0.112 atom per cent deuterium excess, whereas the cholesterol contained 0.336 per cent. The finding that during the experimental period of 22 days the isotope concentration of cholesterol increases almost linearly reflects the slow metabolic turnover of cholesterol, in agreement with earlier results obtained in this laboratory (8).

Feeding of dl-Deuterio Leucine to Rats—Three adult rats having an average weight of 300 gm. were kept on the same low protein (6 per cent casein) diet as described above. In addition, each rat received per day 0.443 gm. of *dl*-deuterio leucine (37.0 atom per cent excess deuterium) and 0.300 gm. of sodium chloride, mixed with the stock diet. The animals were kept on this diet for a total period of 16 days. From the 12th to the 15th day one of the rats was given daily, in addition, 0.300 gm. of *dl*-phenylaminobutyric acid. The urine of this animal was collected and the excreted acetyl-*l*-phenylaminobutyric acid was isolated as described in the foregoing paper (2). It contained 0.50 atom per cent deuterium; *i.e.*, 2.50 per cent in the acetyl group. At the end of the feeding period the three rats were killed; a sample of body water was secured and cholesterol isolated from the pooled carcasses as the digitonide. After decomposition of the digitonide by pyridine-ether (9) 1 1 gm. of cholesterol were obtained. This cholesterol was converted into cholesteryl chloride. The latter contained 0.56 atom per cent deuterium excess. Cholesteryl chloride was degraded into the isooctane-isooctene mixture, as described earlier (1). The hydrocarbon mixture representing the cholesterol side chain contained 0.50 atom per cent deuterium excess and the nuclear hydrocarbon $C_{19}H_{30}$, 0.54 per cent. The average deuterium content of a compound composed of the fragments C_8H_{18} and $C_{19}H_{30}$, as calculated from their isotope content, would be $(18 \times 0.50 + 30 \times 0.54)/48 = 0.53$ per cent. This agrees well with that of the cholesteryl chloride.

Feeding of Deuterio Valine—Two rats weighing about 180 gm. each were kept on the same stock diet which was employed in the leucine experiment. In addition, each rat received daily 0.230 gm. of *dl*-deuterio valine containing 24.7 atom per cent excess deuterium for a period of 8 days. One of the rats was given 0.20 gm. of *dl*-phenylaminobutyric acid per day. Cholesterol was isolated from the carcasses and acetyl-*l*-phenylaminobutyric acid from the urine. The isotope concentrations in the isolated compounds are listed in Table I.

Feeding of Deuterio Isovaleric and Deuterio Isobutyric Acids—The isotopic fatty acids were administered to rats as the sodium salt added to the usual stock diet. The diet further contained 0.100 gm. of *dl*-phenylamino-

butyric acid per 100 gm. of rat weight. The deuterium concentrations of the isolated cholesterol and acetylphenylaminobutyric acids are listed in Table I.

DISCUSSION

In Table I are listed the isotope concentrations in cholesterol and in the acetyl groups of acetylphenylaminobutyric acid excreted after the administration of the labeled test substances. It has been pointed out (1) that the presence in cholesterol of isotope in concentrations greater than half of that of the body fluids constitutes evidence for the utilization of the test substance in cholesterol synthesis. After the administration of deuterio leucine the concentration of isotope in the cholesterol was almost 4 times that in the body fluids; when deuterio isovalerate was fed, the corresponding ratio was nearly 2. The values resulting from the feeding of labeled valine or isobutyric acid are too low to be considered significant. Since leucine was given over twice as long a period as acetate or isovalerate, the relative efficiencies of the various compounds cannot be compared directly. It is permissible, however, to assume that after a period of 8 days the cholesterol in the leucine experiment contained roughly half of the isotope concentration which was found after 16 days.[2] Intermediary acetate formation from leucine and isovaleric acid, which is evident from the high deuterium concentrations in the acetyl groups, can account satisfactorily for the effect given by these compounds in cholesterol synthesis. This relation is illustrated by the ratio of isotope concentrations in the acetyl group to that in cholesterol. The quantities of deuterio acetate formed from leucine and isovaleric acid seem adequate to produce cholesterol with the observed concentrations.

As in experiments in which acetate was fed, the isotope in cholesterol following administration of deuterio leucine is distributed fairly evenly over the side chain and the nucleus. With regard to the possibility that leucine, aside from being a source of acetate, also supplies the isopropyl group of the cholesterol side chain or the angular methyl groups, it must be pointed out that, as the five methyl groups contain only 15 out of a total of 46 hydrogen atoms in the cholesterol molecule, such an effect might not be detectable in our data.

The equivalence of leucine and isovaleric acid with respect to acetate formation supports the hypothesis that degradation to isovaleric acid is a step in the normal metabolism of leucine. The present findings demonstrate the formation of acetic acid as a normal breakdown product of leucine and of isovaleric acid but do not indicate whether acetoacetate is an intermediate   In order to evaluate the analytical data, it will be useful

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[2] This is the case in the experiment in which mice were used.

to consider the coefficient of utilization defined in the foregoing paper (2). This coefficient, which is a measure of efficiency of acetyl formation, when calculated for the 9 carbon-bound hydrogen atoms of isovaleric acid, is 5.1 and has an identical value for the corresponding 9 hydrogen atoms of leucine. Since the coefficient has a value of 15.6 for acetic acid itself, a value of 5.1 for leucine and isovaleric acid is taken to mean that of the 9 hydrogen atoms under consideration 3 appear as acetic acid. These facts, which indicate that 1 mole of acetic acid is formed from leucine, are compatible with the view that isovaleric acid is an intermediate in its degradation. As leucine was administered as the racemic compound, this hypothesis is valid for both isomers. The inversion of *d*-leucine *in vivo* is well established (10). The formation of acetic acid from leucine could conceivably result from either of two processes shown in the accompanying diagram. The steps involved in the degradation of isovaleric acid to acetic

$$CH_3$$
$$\diagdown$$
$$CHCH_2CHCOOH$$
$$\diagup \qquad\qquad NH_2$$
$$CH_3$$

$$CH_3$$
$$\diagdown \qquad \downarrow$$
$$CHCH_2COOH$$
$$\diagup \qquad\qquad \diagdown (B)$$
$$CH_3 \qquad\qquad\qquad \searrow$$

(A) CH_3
$\diagup$ $\diagdown OH$
CH_3COCH_2COOH $C\!-\!CH_2COOH$
$\downarrow$ $\diagup$
$2CH_3COOH$ CH_3

CH_3 $\downarrow$
$\diagdown$
$CO + CH_3COOH$
$\diagup$
CH_3
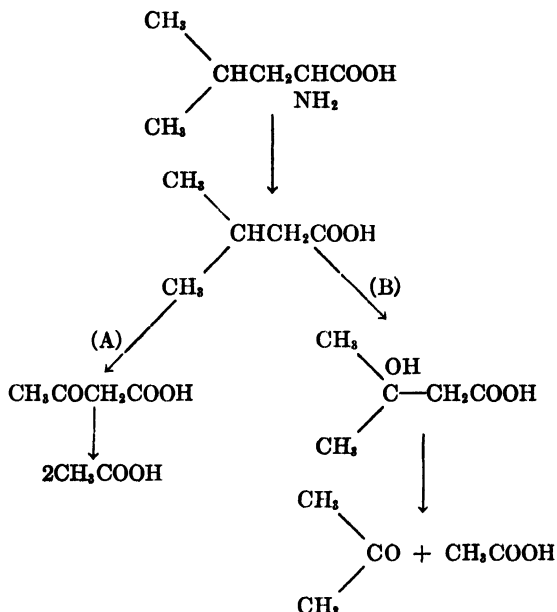
acid must in both cases lead to a loss of carbon-bound deuterium. If demethylation of the isopropyl group takes place and acetoacetate is an intermediate (Pathway A), then isovaleric acid would be expected to yield acetate to the same extent as butyric acid, and it should have a coefficient of utilization intermediate between those found for α,β- and β,γ-dideuterio butyric acids (2). In this case, 5 of the 9 hydrogen atoms, namely those at the α- and γ-carbon atoms, will remain for acetate formation, and on

this basis the coefficient of utilization becomes $9/5 \times 5.1 = 9.18$. If aceto-acetate were the intermediate, the coefficient should fall between the values 3.8 and 7.6 found in Experiments 4 and 5 of the paper just cited.

The second process (Pathway B) postulates the formation of 1 mole each of acetone and acetic acid. According to this scheme the acetoacetic acid arising from isovaleric acid in ketosis would have to be formed by the well recognized (11) condensation of 2 moles of acetate. Only the 2 hydrogen atoms in the α position of isovaleric acid could appear in the acetic acid, and the coefficient of utilization would be $9/21 \times 5.1 = 22.9$, which is greater than that (15.6) calculated for acetic acid itself. Neither one of the two schemes explains satisfactorily the large production of acetate from isovaleric acid. Pathway B would conform with experimental data only if the acetone were to break down *in vivo* to acetic acid. In the absence of such evidence no decision can be made as to whether one of the two pathways mentioned, or a different one, is involved.

The failure of valine and isobutyric acid to yield acetyl as well as cholesterol is consistent with the known glycogenic action of these compounds. According to Rose *et al.* (12), 3 out of the 5 carbon atoms of valine are converted to glycogen. Formation of pyruvate as an intermediary step in this conversion is improbable, since pyruvate is at least partly converted to acetyl. Propionic acid is a possible intermediate in isobutyric degradation, since it is incapable of producing acetic acid (2).

SUMMARY

1. The preparation of *dl*-deuterio leucine, *dl*-deuterio valine, deuterio isovaleric acid, and deuterio isobutyric acid is described.

2. Cholesterol isolated from rats which had received labeled leucine or isovaleric acid contained appreciable concentrations of deuterium, but only normal hydrogen when labeled valine or isobutyric acid was fed.

3. When phenylaminobutyric acid was administered simultaneously with labeled leucine or isovaleric acid, the excreted acetyl derivative of the amino acid contained a high isotope concentration, demonstrating that these two compounds were degraded to acetic acid. Labeled valine and isobutyric acid do not form deuterio acetyl groups. It is suggested that the effect of leucine and isovaleric acid shown in cholesterol synthesis is not specific but due to intermediary acetate formation.

4. From quantitative data it is concluded that isovaleric acid is an intermediate in the oxidative breakdown of leucine.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **145**, 625 (1942).
2. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).
3. Braun, L., *Monatsh. Chem.*, **17**, 213 (1896).
4. Marvel, C. S., in Organic syntheses, New York, **21**, 74 (1941).
5. Marvel, C. S., and du Vigneaud, V., in Organic syntheses, New York, **11**, 20 (1931)
6. Marvel, C. S., in Organic syntheses, New York, **20**, 106 (1940).
7. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
8. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121**, 235 (1937).
9. Schoenheimer, R, and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
10. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940).
11. Swendseid, M. E , Barnes, R H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
12. Rose, W. C., Johnson, T. E., and Haines, W. T., *J. Biol. Chem.*, **145**, 679 (1942).

EFFECT OF IRON ON CARBOHYDRATE METABOLISM OF CLOSTRIDIUM WELCHII*

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During the course of a study on factors underlying growth and toxin production by *Clostridium welchii*, it was observed that, when the concentration of iron in the medium was suboptimal for growth, more acid was produced than when iron was present in excess. This observation was confirmed by rough estimation of the total titratable acid produced from glucose during growth of *Clostridium welchii* in media containing varying amounts of iron and suggested that the nature of the fermentation reaction might be dependent upon the amount of iron present. It was, therefore, of some interest to study the glucose metabolism of this organism in some detail, under conditions in which the sole variable in the medium was iron.

Few careful studies on carbohydrate metabolism of pathogenic bacteria have been reported in the literature. Friedemann and Kmieciak (1) studied the breakdown products from glucose formed after growth of a number of pathogens in complex media. For *Clostridium welchii*, lactic acid, acetic acid, ethyl alcohol, carbon dioxide, and hydrogen were the principal fermentation products and some butyric acid was found. The quantities of these substances which they determined accounted for 60 to 72 per cent of the carbon consumed. Slade *et al.* (2), using washed suspensions of *Clostridium welchii*, also reported ethyl alcohol, lactic, acetic, and butyric acid production from glucose. They did not measure gas production and their data accounted for only 20 per cent of the carbon. In both of the above studies the possible effect of iron on the carbohydrate metabolism was not considered.

Culture Methods

The fermentation of glucose was studied (1) by examining the products formed in whole culture on a medium of defined composition containing varying amounts of iron, and (2) by comparing the products formed from glucose when washed suspensions of organisms grown on a medium deficient in iron are used with organisms cultivated in the same medium containing an excess of iron.

Cultures—Two strains of *Clostridium welchii* were used in this study. The PB6K strain was obtained from the National Institute of Health, and a stock strain of unknown origin was obtained from the Department of Bacteriology, Harvard Medical School. The PB6K strain was maintained by daily transfer in gelatin hydrolysate medium described below. The "Harvard" strain was maintained in chopped meat broth.

Media—Two types of media were used for this study. The first contained a complete acid hydrolysate of purified gelatin as a base, while the other contained casamino acids (Difco) supplemented with tryptone (Difco). The gelatin hydrolysate medium was preferred for the study of products formed in whole cultures, since blank determinations were very small. Somewhat heavier growth occurred with the casein hydrolysate medium which, however, contained a considerable amount of lactic acid, carbohydrate, and other impurities. The casein hydrolysate medium proved satisfactory for preparing washed suspensions of organisms.

Gelatin Hydrolysate Medium—Eastman's "ash-free" gelatin was refluxed for 24 hours with 5 N sulfuric acid and the sulfate removed with barium hydroxide. After the material was decolorized with charcoal, the hydrolysate was made up as a stock solution so as to contain 22 mg. of nitrogen per cc.

200 cc. of stock gelatin hydrolysate, 25 mg. of tryptophane, 100 mg. of tyrosine, 200 mg. of dl-methionine, 3 gm. of glutamic acid, 10 gm. of sodium glycerophosphate, and 1 gm. of potassium acid phosphate are brought to about 500 cc. with distilled water and the pH adjusted to 7.6 with 5 N sodium hydroxide. 2 cc. of a 10 per cent solution of calcium chloride are added and after the preparation is heated to boiling the precipitated calcium phosphate is filtered and the filtrate tested for iron with α,α'-bipyridine. If any iron is present, the calcium phosphate precipitation is repeated until no appreciable pink color is formed with the bipyridine reagent (less than 0.04 γ of iron per cc. as determined in the Coleman spectrophotometer). After the material is cooled, 10 mg. of adenine sulfate, 10 mg. of uracil, 1 cc. of salt mixture (containing 50 mg. of copper sulfate ($CuSO_4 \cdot 5H_2O$), 50 mg. of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), and 20 mg. of manganese chloride ($MnCl_2 \cdot 4H_2O$) per 100 cc.), 1 cc. of 10 per cent magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.2 cc. of 20 per cent cystine hydrochloride, and 1 cc. of accessory factors (20 mg. of nicotinic acid, 20 mg. of calcium pantothenate, 20 mg.

of pyridoxine, 10 mg. of thiamine, 5 mg. of riboflavin, and 0.01 mg. of biotin per 20 cc.)[1] are added and the medium made up to such a volume that the addition of the remaining components will bring it to exactly 1 liter. The medium is then distributed in 4 ounce acid-cleaned bottles and autoclaved at 10 pounds pressure for 10 minutes. Before inoculation 10 cc. of sterile 10 per cent glucose, 1 cc. of 1 per cent thioglycolic acid, 1 to 5 cc. of a solution containing the desired amount of ferrous sulfate ($FeSO_4 \cdot 7H_2O$), and 2.5 cc. of a 6 hour culture of the PB6K strain of *Clostridium welchii* grown on the same medium are added to bring the volume to 100 cc. per bottle.

Casein Hydrolysate-Tryptone Medium—20 gm. of casamino acids (Difco), 20 gm. of tryptone (Difco), 6 gm. of sodium glycerophosphate, 4 gm. of potassium acid phosphate, 200 mg. of magnesium sulfate, 2 gm. of sodium chloride, and 0.3 cc. of 20 per cent cystine hydrochloride are brought to 1 liter with distilled water and adjusted to pH 7.6 to 7.8. 4 cc. of 10 per cent calcium chloride are added, and the medium brought to boiling and filtered. The calcium chloride treatment is repeated if necessary until a negative test for iron is obtained with bipyridine. The medium is then made up to 2 liters with distilled water and 2 cc. of addition mixture containing the B vitamins listed above are added. 800 cc. of medium are placed in each of two acid-cleaned 1 liter Erlenmeyer flasks and the remaining medium is distributed in test-tubes measuring 2.5 by 15 cm. Before inoculation, 10 cc. of 50 per cent glucose, 0.1 cc. of thioglycolic acid, and the desired quantity of ferrous sulfate are added for each liter of medium. The inoculum consists of 10 cc. of a 6 to 9 hour culture of *Clostridium welchii* grown on the same medium.

Estimation of Growth—Growth was measured by turbidity in the Coleman universal spectrophotometer at 650 mμ and estimated as mg. of bacterial nitrogen per 100 cc. from a curve plotted with dilutions of a standard suspension of known bacterial nitrogen content.

Determination of Iron in Culture Medium—The pink color developed with bipyridine after reduction with sodium hydrosulfite or ascorbic acid was measured in the spectrophotometer at 515 mμ.

Analytical Methods

Gas Production—Carbon dioxide and hydrogen were determined separately in the Warburg apparatus. To each vessel were added 1 cc. of a suspension of *Clostridium welchii* containing 0.16 to 0.2 mg. of bacterial nitrogen per cc., 0.5 cc. of 0.2 M phosphate buffer at pH 7, and sufficient distilled water so that the total volume including that in the side arms was exactly 2.1 cc. After equilibration under oxygen-free nitrogen in the

water bath at 35°, 0.01 mM of glucose was tipped in from one side arm. Hydrogen was estimated after the carbon dioxide evolved was absorbed with 20 per cent sodium hydroxide placed in the center cup. Carbon dioxide was determined by subtracting the hydrogen evolved from the total gas produced, including the carbon dioxide liberated by tipping in 0.1 cc. of 40 per cent phosphoric acid from one side arm at the end of the experiment. Correction was made for initially bound carbon dioxide. The residual liquid was analyzed for lactic acid colorimetrically and the values so obtained closely checked those from the macro experiments. Control vessels without glucose consistently gave negative results.

Lactic Acid—In the experiments with washed organisms, lactic acid was determined colorimetrically with *p*-hydroxydiphenyl according to the method of Barker and Summerson (3). The color was read in the spectrophotometer at 560 mμ. Lactic acid in the culture supernatants was determined by the Friedemann and Graeser method (4) with the apparatus described by West (5). Recovery of lactic acid was about 96 per cent by this method and duplicates usually checked within 2 to 5 per cent.

Residual Glucose—Glucose was determined by the Stiles, Peterson, and Fred modification of the Shaffer-Hartmann method (6).

Volatile Acids—A suitable aliquot was acidified to Congo red with phosphoric acid and was steam-distilled until the distillate no longer contained significant quantities of acid. The total volatile acids were estimated with 0.04 N sodium hydroxide and after they were concentrated to a small volume on the water bath the solution was again acidified and redistilled with steam. The individual acids were then determined by partition in isopropyl ether by the method of Osburn and Werkman (7).

Alcohol—Suitable aliquots of the fermentation mixture were distilled over mercuric oxide (HgO) and mercurous sulfate (Hg_2SO_4). The distillate was made alkaline to brom-thymol blue and redistilled over mercuric oxide into a volumetric flask. Aliquots of the final distillate were treated with standard potassium dichromate at 85° for 1 hour and the equivalents of dichromate used up were determined by titration with thiosulfate with potassium iodide and starch as indicator. No attempt was made to identify the individual alcohols and the results were calculated as ethyl alcohol.

Experiments and Results

Iron Content of Clostridium welchii Cells—During growth of *Clostridium welchii* the iron present in the culture is taken up quantitatively by the cells and no iron can be detected in the culture supernatant until its concentration surpasses the optimum for growth. Table I shows the effect of adding iron to casamino acid-tryptone medium containing 0.4 per cent maltose.

It will be noted that no iron could be detected in the culture supernatant until its concentration was in excess of about 0.6 mg. per liter and that the iron content of the cells varied from about 0.0005 mg. of iron per mg. of bacterial nitrogen to a maximum of 0.0039 mg. of iron per mg. of bacterial nitrogen (0.007 to 0.05 per cent iron on a basis of dry weight of bacteria). The organisms themselves turn bright pink when treated with bipyridine. Since these results were obtained, data on the assimilation of iron by various other species have been reported by Waring and Werkman (8).

Effect of Iron on Lactic Acid Production—These experiments were carried out with gelatin hydrolysate medium containing 0.8 and 1.0 per cent glucose, from which the iron had been removed as completely as possible (that is, had been reduced to about 0.03 to 0.05 mg. per liter). Before

TABLE I

Effect of Increasing Iron Content of Medium on Iron Content of Clostridium welchii Cells

Casamino acid-tryptone medium containing 0 4 per cent maltose.

Iron content of medium	Iron in culture super-natant	Growth, bacterial N per 100 cc	Iron content of bacteria, Fe per mg bacterial N
mg per l.	*mg. per l.*	*mg.*	*mg.*
0.04 (*Ca.*)	<0.03	8.2	0.0005 (*Ca.*)
0.13	<0.03	13.3	0.0010
0.23	<0.03	14.3	0.0016
0.33	<0.03	16 0	0.0021
0.43	<0 03	16.2	0.0027
0.53	<0 03	16 8	0.0032
0.73	0.13	16 7	0.0038
0 93	0 27	16.9	0.0039
1.13	0 50	16.0	0.0039
2.13	1 47	17.0	0.0039

inoculation, ferrous sulfate equivalent to 0.0, 0.1, 0.5, 1.0, and 2.0 mg. of iron per liter was added to each of a series of bottles containing the medium. After 36 hours incubation at 37°, the growth, residual glucose, and lactic acid were determined for each culture. Fig. 1 shows the effect of iron on lactic acid production. Each point represents the average of more than four separate experiments. The points at zero iron added represent results obtained after removal of as much of the iron from the medium as possible. It can be seen that as the iron content is decreased the lactic acid production approaches 2 moles per mole of glucose fermented. This limit is a hypothetical one, since no growth occurs in the complete absence of iron.

Fermentation of Glucose by Washed Organisms—Complete carbon balances

from glucose were carried out in duplicate with washed suspensions of *Clostridium welchii* grown in media containing high and low concentrations of iron. The details of only one complete experiment will be given here.

Two flasks, A and B, each containing 800 cc. of casamino acid-tryptone medium were inoculated with 10 cc. of a 12 hour culture of the "Harvard" strain of *Clostridium welchii* grown on the same medium. Before inoculation 0.1 cc. of thioglycolic acid and 8 cc. of 50 per cent glucose were added to each flask. The iron content of Flask A was approximately 0.04 mg. of iron per liter. 5 cc. of 0.1 per cent ferrous sulfate were added to Flask B (1.25 mg. of iron per liter). After 12 hours at 35° the flasks were removed from the incubator. The pH of Flask A was 4.7 and the growth equivalent
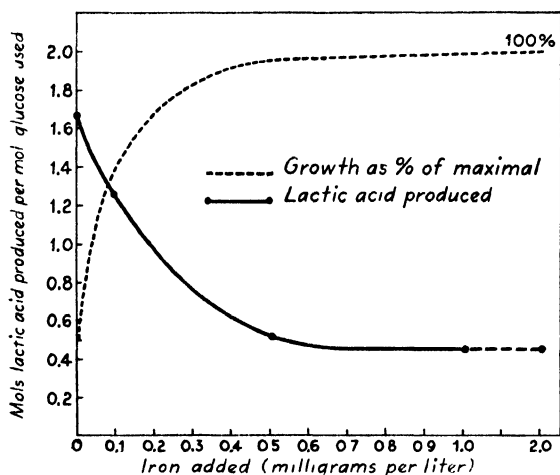
FIG. 1. The dotted line represents the growth of organisms as a percentage of the maximum growth obtainable with excess iron in the medium The solid line represents moles of lactic acid produced per mole of glucose utilized

to 8.2 mg of bacterial nitrogen per 100 cc. The pH of Flask B was 4.9 and the growth 14.0 mg. of bacterial nitrogen per 100 cc. Each culture was centrifuged in acid-cleaned Pyrex vessels and washed once with boiled saline, after which the organisms with low iron content were made up to a turbidity equivalent to 2.58 mg. of bacterial nitrogen per cc. (Suspension A) and with high iron to 2.62 mg of bacterial nitrogen per cc. (Suspension B). The suspensions were used immediately both in the Warburg experiments as described and in macro experiments as described in the following.

The fermentations were carried out in 250 cc. conical flasks fitted with a ground glass stopper through which were sealed two glass tubes, one of

which passed to the bottom. Both tubes were fitted with stop-cocks. To Flask A were added exactly 50 cc. of 0.2 M phosphate buffer at pH 7, 5 cc. of 0.4 M glucose (2.0 mM), 10 cc. of Suspension A, and 35 cc. of distilled water. To Flask B were added 50 cc. of 0.2 M phosphate buffer, 10 cc. of 0.4 M glucose, 15 cc. of Suspension B, and 40 cc. of distilled water. Both flasks were then flushed out for 5 to 10 minutes with oxygen-free nitrogen and then quickly evacuated on the water pump. The stop-cocks were closed and the fermentation was allowed to proceed for 20 hours at 35°.

TABLE II

Effect of Iron on Glucose Fermentation by Washed Suspensions

	Low iron		High iron	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Bacterial iron content (Fe per mg bacterial N), *mg*..	0.0005 (*Ca*)	0.0005 (*Ca*.)	0 0039	0.0039
Glucose fermented, *mM*	1 00	1.00	1 00	1.00
Lactic acid, *mM*	1.73	1.60	0.42	0.33
Total volatile acids, *mM*	0 06	0 24	0 88	0.94
Acetic acid, *mM*	(0 04)	(0.15)	0 56	0.60
Butyric " " .. .	(0.02)	(0.09)	0.32	0.34
Alcohol (as ethanol), *mM*	0 025	0 10	0.16	0.26
Carbon dioxide, *mM*	0 33	0 24	1 35	1 76
Hydrogen, *mM*	0 38	0 21	1.93	2.14
Carbon recovery, %	95 5	98	89 2	97 3

The results are given as mM of product per mM of glucose fermented. Hydrogen and oxygen recoveries from low iron fermentation were 96 to 100 per cent. The hydrogen and oxygen recoveries with high iron, after correction for lactic acid production, were greater than 100 per cent and indicated that 1 molecule of water enters into the acetic-butyric acid fermentation   The values in parentheses were uncertain owing to the small quantities involved

At the same time similar buffered suspensions were incubated without glucose, to serve as controls.

After 20 hours incubation, 10 cc. of 30 per cent metaphosphoric acid were added to each flask. The suspensions were centrifuged and each supernatant was collected and analyzed for residual glucose, lactic acid, volatile acids, and alcohol. The results of two complete experiments are shown in Table II. The results from the control determinations without glucose are not included in Table II since the yields in every case were too small to be of significance. The yields of lactic acid from glucose produced by washed suspensions are in good agreement with those obtained from whole cultures. Thus, with cells grown on a medium containing excess iron, yields of 0.33 and 0.42 moles of lactic acid per mole of glucose were ob-

tained. The average of seventeen experiments on supernatants of whole cultures yielded 0.46 mole with a variation from about 0.3 to 0.5 mole of lactic acid produced per mole of glucose fermented. The iron-deficient cells yielded 1.6 and 1.73 moles of lactic acid per mole of glucose fermented, compared with an average of 1.66 moles produced by four cultures grown in deferrated gelatin hydrolysate medium. From preliminary results it would appear that volatile acid production by whole cultures will also check the results with washed suspensions, suggesting that carbohydrate fermentation is the sole source of these products in the defined medium.

With the exception of butyric acid production, the yields of the various products from glucose by organisms of high iron content agree fairly well with those reported by Friedemann and Kmieciak (1). The medium used by them apparently contained close to the optimal iron concentration. Friedemann and Kmieciak's yields of butyric acid were very much lower than ours.

<center>DISCUSSION</center>

According to Kubowitz (9), all fermentations in which free hydrogen is concerned are inhibited by carbon monoxide. In particular, Kubowitz showed that the fermentation of glucose by washed suspensions of *Clostridium butyricum*, from which acetic and butyric acids are normally produced, is shifted towards a pure lactic acid type of fermentation when the process is allowed to take place under carbon monoxide. Relatively high concentrations of cyanide (10^{-2} M) tend to cause a shift in metabolism in the same direction. The results here reported show that a similar change in metabolism of *Clostridium welchii* may be brought about by reducing the iron content of the bacteria. Provided the medium contains an excess of iron, the principal fermentation products from glucose are acetic, butyric, and lactic acids, ethyl alcohol, and considerable quantities of carbon dioxide and hydrogen. On the other hand, when *Cl. welchii* is grown on a medium deficient in iron, the fermentation process is a far less efficient one and lactic acid is the chief end-product   Organisms of low iron content produce minimal quantities of gas and of acetic and butyric acids, whereas the yield of lactic acid from glucose under these conditions may be increased more than 5-fold. Since lactic acid is an end-product and since added lactic may be recovered unchanged from cultures of *Cl. welchii* grown in the presence of excess iron, it would appear that two separate mechanisms exist for the breakdown of glucose. One of these would require the presence of a considerable amount of an iron-containing enzyme. Both mechanisms presumably act simultaneously, but the extent to which a given pathway is followed is dependent upon the concentration of iron-containing enzyme within the cell   In all probability, it would be this

iron-containing enzyme which is inhibited by carbon monoxide and cyanide in the experiments of Kubowitz referred to above.

Glucose ⟶ intermediate ⟶ 2 lactic acid

               Fe ⟍ enzyme

                  Acetic acid
                  Butyric acid
                  Alcohol
                  Hydrogen
                  Carbon dioxide
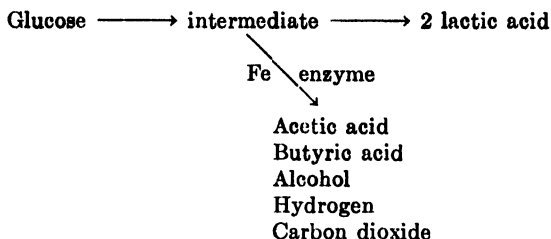
Further work is necessary to determine the existence and nature of such an iron-containing enzyme and the reaction which it catalyzes. The intermediate in the accompanying scheme may well be pyruvic acid or a derivative thereof, since Kubowitz (9) demonstrated that the same shift towards lactic acid production under carbon monoxide occurs with pyruvate as substrate as with glucose.

It is generally supposed that strict anaerobes, such as the *Clostridia*, do not possess the cytochrome system (10). The enzyme postulated above must, therefore, contain iron in some other form. There is some evidence that the iron is loosely bound in the postulated enzyme in a manner comparable to magnesium in the thiamine-containing enzymes. In the first place, lactic acid and gas production by washed organisms, as well as growth in defined media, are completely inhibited by 0.002 M bipyridine This suggests that iron is not present as a hemin derivative. It also infers that iron may enter into some other step in the scheme of glucose breakdown besides the reaction leading to acetic and butyric acid formation. This inhibitory action of bipyridine on *Clostridium welchii* stands in contrast to its effect on aerobic organisms known to contain the cytochrome system. Growth and metabolism of the diphtheria bacillus, for example, are apparently unaffected by bipyridine.[2] Secondly, the acetic acid-butyric acid fermentation by washed suspensions of *Clostridium welchii* is relatively insensitive to cyanide and the shift to the lactic acid type of fermentation is only partial even in the presence of 0.001 M potassium cyanide. Finally, preliminary experiments have indicated that the same effect does not occur when inorganic iron is replaced by hemin or by other metals such as manganese and copper.

When iron is added to washed suspensions deficient in iron, lactic acid remains the chief end-product. However, as the fermentation proceeds, there is a small but definite acceleration in the carbon dioxide and hydrogen production and the final yield of lactic acid is somewhat lowered.

[2] Pappenheimer, A. M., Jr , unpublished experiments.

One of the common reactions used for the identification of *Clostridium welchii* is its ability to produce the so called "stormy fermentation" in milk. In order to obtain consistent and characteristic reactions, the addition of iron has been recommended (11). The significance of this finding is clear from the present results, since milk is known to be deficient in iron.

So far as we are aware, there have been no previous studies on the effect of iron on carbohydrate metabolism of pathogenic bacteria reported in the literature. It has been recently shown that carbohydrate utilization and gas formation by a strain of the tetanus bacillus are increased with increasing iron concentration in the medium.[3] On the other hand, it has been known for some time that traces of iron exert an important influence on the yields of various other metabolic products, notably bacterial toxins. Thus maximum yields of porphyrin and toxin by the diphtheria bacillus (12) and of tetanus toxin by most strains of the tetanus bacillus (13) are obtained only under conditions of relative iron deficiency. In the present experiments with *Clostridium welchii*, iron concentrations necessary for maximum toxin (lecithinase) production, maximum growth, and minimum lactic acid production coincided. Provided the medium is suitable for toxin production, the yield of toxin or lecithinase closely parallels the change in metabolism. The relationship between the lecithinase production and carbohydrate metabolism is not a direct one, however, since the shift to a lactic acid type of fermentation at low iron concentration occurs equally well under conditions otherwise unsuitable for lecithinase production (that is, in the absence of toxin-promoting factor).

It may be remarked in closing this discussion, that carbohydrate metabolism, lecithinase production, and growth of *Clostridium welchii* are not the only factors dependent upon the concentration of iron in the medium. The morphology of the organisms is markedly altered as well. Organisms of low iron content are elongated, curved, and entirely atypical. As their iron content increases, there is a gradual transition to the typical morphological structure, which parallels the altered metabolism. Further work is in progress on this phase of the problem.

SUMMARY

The products obtained from the breakdown of glucose by *Clostridium welchii* depend upon the iron content of the cells.

As the iron content is decreased, the reaction shifts from a predominantly acetic-butyric acid type with production of large amounts of carbon dioxide and hydrogen towards a more purely lactic acid type of fermentation with

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[3] Mueller, J H , and Pickett, M. J., personal communications.

slight gas formation. Under the conditions of these experiments the iron concentration necessary for maximum growth, optimum toxin production, and minimum lactic acid production is identical. A progressive change in morphology accompanies decreased cellular iron content and parallels the change in metabolism.

One of us (A. M. P., Jr.) is greatly indebted to Dr. Morris J. Pickett of the Department of Bacteriology, Harvard Medical School, without whose advice and assistance this work would not have been completed.

BIBLIOGRAPHY

1 Friedemann, T. E., and Kmieciak, T. C , *Proc. Soc. Exp. Biol. and Med.*, **47,** 84 (1941).
2. Slade, H. D , Wood, H G., Nier, A O , Hemingway, A., and Werkman, C H., *J. Biol Chem* , **143,** 133 (1942).
3 Barker, S. B., and Summerson, W. H , *J. Biol. Chem.*, **138,** 535 (1941).
4 Friedemann, T. E , and Graeser, J B , *J. Biol. Chem.*, **100,** 291 (1933).
5 West, E S., *J Biol Chem.*, **92,** 483 (1931).
6 Stiles, H. R., Peterson, W. H , and Fred, E. B., *J. Bact* , **12,** 427 (1926).
7 Osburn, O. L , and Werkman, C. H , *Ind and Eng Chem* , *Anal. Ed* , **3,** 264 (1931)
8 Waring, W S , and Werkman, C. H., *Arch. Biochem* , **1,** 303, 425 (1943).
9 Kubowitz, F , *Biochem Z* , **274,** 285 (1934)
10 Stephenson, M , Bacterial metabolism, London, New York, and Toronto, 2nd edition, 29 (1939)
11 Hastings, E G , and McCoy, E , *J Bact* , **23,** 54 (1932)
12 Pappenheimer, A M , Jr , and Johnson, S J , *Brit J Exp. Path* , **18,** 239 (1937)
13 Mueller, J H., Schoenbach, E B , Jezukawicz, J J , and Miller, P A , *J Clin. Invest* , **22,** 315 (1943)

THE DETERMINATION OF PHENYLALANINE IN PROTEINS*

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In an attempt to use the modification of the Kapeller-Adler reaction (1) for the determination of phenylalanine, described by Block and coworkers (2, 3), we have found, in agreement with Knight and Stanley (4), that tryptophane interferes. These authors corrected for the interference by subtraction from the colorimeter reading of the unknown a value corresponding to the amount of tryptophane present in the aliquot of solution taken for analysis. In our experience the amount of color produced by phenylalanine may vary somewhat from day to day, so that for greatest accuracy it would be necessary to redetermine the correction for tryptophane each time phenylalanine is determined.

The use of acid for hydrolysis might at first glance appear to be desirable, in that tryptophane would be destroyed simultaneously. Block *et al.* (3) have pointed out that phenylalanine is destroyed to a certain extent when acid is used for hydrolysis. But even if this loss of phenylalanine by acid hydrolysis is taken into consideration, their results for different hydrolytic agents recalculated on this basis are not in agreement with each other. This probably indicates that the destruction of tryptophane was neither uniform nor complete in all cases.

A more desirable method would include the removal of tryptophane from the aliquots of the protein hydrolysate before the phenylalanine is nitrated. It appeared that this might be accomplished satisfactorily by means of the well known precipitation with mercuric sulfate.

EXPERIMENTAL

Reagents—

Sodium hydroxide, approximately 5 N.

Kaolin.

Sulfuric acid, approximately 7 N.

Mercuric sulfate solution, 15 per cent; 30 gm. of mercuric sulfate are placed in a 200 ml. flask and 80 to 90 ml. of 7 N sulfuric acid are added, followed by 30 ml. of distilled water. The flask is shaken until the solid is completely dissolved and the solution is then made to 200 ml. with 7 N sulfuric acid. The solution may be filtered if necessary.

Celite Analytical Filter-Aid, Johns-Manville.

Nitrating mixture. Dissolve 20 gm. of powdered potassium nitrate in 100 ml. of concentrated sulfuric acid.

Hydroxylamine hydrochloride; 30 gm. of hydroxylamine hydrochloride dissolved in 100 ml. of distilled water.

Ammonium hydroxide, concentrated.

Procedure

A gm. of protein is hydrolyzed under a reflux in an oil bath at 125° with 16 ml. of 5 N sodium hydroxide. A 2.5 × 20 cm. Pyrex test-tube fitted with a "cold finger" made of a 1:5 × 15 cm. test-tube, rubber stopper, and glass tubing is used for this purpose (5, 2). In order to obtain complete hydrolysis the solution is heated for 24 hours. If peptides are present, these are also precipitated by mercuric sulfate. At the end of the period of hydrolysis, 24 ml. of 7 N sulfuric acid are slowly added to the hot solution with stirring. The condenser is washed down and the amino acid solution is transferred to a 100 ml. volumetric flask. After the cooled solution is made to volume with distilled water, 400 mg. of kaolin are added, the suspension is shaken well, and the solution is centrifuged and filtered through dry filter paper. If filtration is slow, the filter is covered with a watch-glass to prevent evaporation.

Four aliquots of the filtrate, containing approximately 1.5 to 2 mg. of phenylalanine, are pipetted into 40 ml. graduated centrifuge tubes. Water is added to the 20 ml. mark. Then 6 ml. of the 15 per cent mercuric sulfate solution are added and the tubes are placed in a gently boiling water bath for 10 minutes. The solutions are allowed to cool and 4 ml. of 7 N sulfuric acid are added. The solutions are diluted to the 40 ml. mark with water, 20 mg. of Celite are added, and the contents mixed and centrifuged for 5 minutes. The supernatant solution is decanted carefully from the tryptophane-mercuric sulfate complex and Celite into a 50 ml. centrifuge tube, drained, and the lip rinsed with a few drops of distilled water.

The mercury is precipitated from the supernatant solution with hydrogen sulfide, centrifuged, and filtered. The precipitate remaining in the centrifuge tube is mixed with 12 ml. of distilled water, a drop of 7 N sulfuric acid added, and hydrogen sulfide is bubbled through again. After the precipitate is centrifuged, the washings are decanted through the same filter.

The excess sulfuric acid is removed by the addition, with stirring, of about 8 gm. of barium hydroxide (octahydrate) dissolved in 10 ml. of hot distilled water. The solution should remain acid to Congo red paper. By using 1 × 8 mm. strips of indicator paper, losses of phenylalanine absorbed by the paper are insignificant. At this point the volume should be small enough for the solution and precipitate to be transferred quantitatively into

a 100 ml. centrifuge tube. After being centrifuged, the supernatant solution is decanted through a filter. The residue of barium sulfate is rubbed and washed thoroughly with 50 ml. of hot distilled water containing a drop of sulfuric acid and a drop of capryl alcohol. The tubes are centrifuged and the supernatant solution decanted through the filter. The washing and centrifuging are repeated with 40 ml. of hot distilled water and a drop of 7 N sulfuric acid.

The filtrate and washings of each aliquot are evaporated to dryness in 7 cm. porcelain dishes on the steam bath. After being cooled, 4 ml. of the nitrating mixture are added to each dish and the dishes are warmed on the steam bath for 20 minutes. While still hot, the nitrated solutions are transferred to 50 ml. stoppered graduated Pyrex cylinders. If small portions of water are used for washing, the final volume in the cylinders can be kept to a maximum of 18 to 20 ml.

The solutions are cooled in ice water to 0°. When cold, 5 ml. of 30 per cent hydroxylamine hydrochloride are added to three of the graduates. One aliquot of each set, to which no hydroxylamine hydrochloride is added, is used as a protein blank. The solutions are again cooled in ice water for 1 minute, and all are diluted to the 50 ml. mark with ice-cold concentrated ammonium hydroxide. If the ammonia is added slowly down the side of the graduate, this can be accomplished without danger, but great care must be exercised. The solutions are mixed and the color is allowed to develop at room temperature for 45 minutes. Before the end of this period, but after the solutions have come to room temperature, the final adjustment in volume is made. The colored solutions may be filtered through fast, dry paper if a precipitate is formed.

The transmission of the color developed is determined at λ 560 mμ with a Coleman No. 11 spectrophotometer. The solution to which no hydroxylamine hydrochloride was added is used as the blank. Photoelectric colorimeters with 560 mμ filters should be satisfactory also.

It is advisable to determine the factor for the color produced by phenylalanine each time determinations of phenylalanine in protein are made. Aliquots of standard phenylalanine solution containing approximately the quantity expected in the aliquots of protein hydrolysate have proved to be quite satisfactory for this purpose. Summerson (6) has called attention to the desirability of this procedure over that of reading from a curve prepared some time earlier.

Recovery of Phenylalanine—A mixture of amino acids having the composition shown in Table I was prepared. To aliquots of this mixture were added 2 mg. of phenylalanine and, in some cases, tryptophane. The nitration and development of color were carried out in the usual manner. The results obtained are shown in Table II. It is obvious that in the presence

of tryptophane an increase in color value related directly to the amount of tryptophane present is obtained. Satisfactory recoveries of phenylalanine are obtained when the added tryptophane is removed as outlined.

Purified gelatin was used for checking losses in the process for the separation of tryptophane from phenylalanine because this protein contains, at most, only a trace of tryptophane. The gelatin was hydrolyzed with 5 N

TABLE I

*Composition of Amino Acid Mixture**

	gm.		gm.
dl-Alanine	0 2161	dl-Threonine	0 1203
l-Aspartic acid	0 2872	d-Arginine monohydrochloride	0 5092
l-Tyrosine	0 2771	dl-Serine	0 0952
dl-Methionine	0 0335	l-Leucine	0 1212
l-Cystine	0.0730	l-Histidine dihydrochloride	0 0917

* Heated with 30 ml. of 5 N sodium hydroxide under a reflux at 130° for 3 hours, added to 45 ml. of 7 N sulfuric acid, and made to 200 ml

TABLE II

Recovery of Phenylalanine from Synthetic Mixtures

Amino acids taken		Phenylalanine recovered	
		Tryptophane not removed	Tryptophane removed
	mg.	mg.	mg.
Amino acid mixture	25.5	2.00	
Phenylalanine	2.0		
Amino acid mixture	25.5		
Phenylalanine	2.0	2 50	2 05
Tryptophane	0.4		
Amino acid mixture	25.5		
Phenylalanine	2.0	2 80	2.06
Tryptophane	0 8		

sodium hydroxide and the hydrolysate prepared for analysis as described. Three series of determinations of phenylalanine were made. In the first series, the determinations were made according to Block and Bolling (2), without further modification. In the second, the process for the removal of tryptophane as outlined above was applied and the excess of sulfuric acid was removed with barium hydroxide. Barium carbonate was used in place of barium hydroxide to remove the excess sulfuric acid in the third series of determinations. 1.39, 1.34, and 1.70 per cent respectively of phenylalanine

were found for the three series. As shown, there is no significant loss of
phenylalanine when the determination is carried out as outlined. The
values obtained when barium carbonate is used to remove the excess sulfuric
acid are too high. This observation although repeatedly made was not
investigated further. It does show, however, that it is desirable that the
barium hydroxide used be low in carbonate. Erratic results have been ob-
tained when old barium hydroxide containing considerable carbonate was
used.

TABLE III

Phenylalanine Content of Proteins

Prepara-tion No *	Protein	Phenyl-alanine content	Values from literature and bibliographic reference No.
		per cent†	*per cent*
1	Arachin	5.03	2 60 (7)
2	Conarachin	3.29	
3	Water-dispersible peanut protein	4 56	
4	Casein	3 99	3 88 (8), 3 70 (9), 5 8 (2), 3 2 (10)
5	Lactalbumin	3 63	2 4 (11), 1 25 (12), 4 8 (2)
6	Gelatin	1 57	1 15 (1), 1 4 (13), 1 8 (14), 2 6 (3)
7	Hair (human)	1 80	2 7 (15)
8	" "	1 90	

* Preparations 1 and 2, arachin and conarachin described earlier (16), Preparation
3, water-dispersible peanut protein described earlier (17); Preparation 4, Labco
vitamin-free casein; Preparation 5, Labco 7-HAAX lactalbumin; Preparation 6,
Eastman Kodak Company purified calf skin gelatin; Preparation 7, human hair,
brown, 14 year-old female; suspended in distilled water at room temperature for 12
hours, dried on filter paper for 12 hours, then extracted in a continuous extraction
apparatus with 95 per cent alcohol, ether, and then petroleum ether for 48, 24, and 24
hours, respectively; Preparation 8, human hair, gray, 60 year-old female, treated as
Preparation 7.

† Averages of several determinations corrected for moisture and ash.

Results

In Table III are shown the results obtained for phenylalanine on eight
protein preparations by the modification reported here. Included for com-
parison are the results found by several other investigators. The result
reported here for casein agrees well with those reported by Foreman (8) and
by Hegsted (9). The latter value was obtained by microbiological assay.
Determinations of phenylalanine made without removal of the tryptophane
gave results which, with the exception of gelatin, in general agreed with
those of Block and Bolling (2, 15).

SUMMARY

1. The presence of tryptophane in a mixture of amino acids or in a protein hydrolysate interferes in the determination of phenylalanine by the Kapeller-Adler reaction. When the tryptophane is removed by precipitation with mercuric sulfate solution before nitration, reliable results are obtained for phenylalanine.

2. The results for phenylalanine obtained by the modified method on eight protein preparations are reported.

BIBLIOGRAPHY

1. Kapeller-Adler, R , *Biochem Z* , **252,** 185 (1932)
2. Block, R. J , and Bolling, D , The determination of the amino acids, Minneapolis, revised edition (1940)
3 Block, R J , Jervis, G A , Bolling, D , and Webb, M , *J Biol. Chem* , **134,** 567 (1940)
4. Knight, C A , and Stanley, W M , *J Biol Chem* , **141,** 39 (1941)
5 Folin, O , and Marenzi, A D , *J Biol Chem* , **83,** 89 (1929)
6 Summerson, W H , *J Biol Chem* , **130,** 149 (1939)
7. Johns, C O , and Jones, D B , *J Biol. Chem* , **36,** 491 (1918)
8. Foreman, G W , *Biochem J* , **13,** 378 (1919)
9 Hegsted, D M , *J Biol. Chem* , **152,** 193 (1944)
10 Abderhalden, E , *Z physiol Chem* , **44,** 23 (1905)
11 Abderhalden, E , and Pribram, H , *Z physiol Chem* , **51,** 409 (1907).
12 Jones, D B , and Johns, C O , *J Biol Chem* , **48,** 347 (1921)
13 Dakin, H D , *J Biol Chem* , **44,** 499 (1920)
14 Gordon, A H , Martin, A J P , and Synge, R L M , *Biochem J* , **37,** 92 (1943)
15. Block, R J , and Bolling, D , *Arch Biochem* , **3,** 217 (1943)
16. Brown, W L , *J Biol Chem* , **142,** 299 (1942)
17 Brown, W. L , *J Biol Chem* , **154,** 57 (1944)

WATER-SOLUBLE VITAMINS IN HAIR AS INFLUENCED BY DIET

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The first part of the present paper deals with a method for determining certain water-soluble vitamins in hair, and the second part concerns variations in the concentration of these vitamins in the hair as influenced by diets low in one or more of such vitamins.

Methods

No method for the determination of water-soluble vitamins in hair appears to have been previously reported. The special problem presented in the case of hair is that of breaking it up so that the soluble constituents can be extracted without destroying any of the vitamins. One type of procedure that suggested itself was actually to dissolve the keratin of the hair by the use of solutions of sodium sulfide, potassium cyanide, or thioglycolic acid at alkaline pH, as shown by Goddard and Michaelis (1). This procedure did not fit in very well with microbiological determinations of the vitamins. Resort was therefore had to grinding, and the use of the ball mill for this purpose was studied. Routh and Lewis (2, 3) found that ground wool was digested by trypsin and that some oxidation of the sulfur of the wool occurred on prolonged grinding. We have studied the use of the ball mill as a preliminary procedure in the analysis of hair for water-soluble vitamins. The following procedure was found to be most satisfactory.

Procedure—The hair is washed with cold water and dried at about 60°. From 0.5 to 1.0 gm. of the washed dried hair is ground in a small ball mill at 75 R.P.M. for 3 days. In this way from 95 to 100 per cent of the hair becomes soluble. An appreciable amount of inorganic matter is added from the stones. A suitable amount of dried ground hair (usually from 0.4 to 1.0 gm.) is shaken with hot water and then filtered. The residue on the filter paper is washed twice with hot water, the washings and filtrate combined, cooled, and made up to 40 or 50 cc. One-fourth of the solution is kept for the nicotinic acid determination (4). The remainder is extracted

with an equal volume of ether to remove fatty acids, which have been shown to interfere with assay methods (5). The aqueous ether-extracted solution is analyzed for riboflavin (6), pantothenic acid (7), and inositol (8), with 0.5, 1, 3, and 5 cc. samples. A portion of the ground hair is ashed in a crucible and loss of weight determined so that a correction can be made for inorganic matter added from the stones.

Discussion of Method—The influence of washing the hair with water, alcohol, and ether before grinding was studied, as well as the effect of washing the ground material with alcohol before analysis. Table I shows no appreciable effect of either procedure on the results, so that no appreciable loss of these vitamins from the hair by washing seems likely and the vitamins found can hardly be in appreciable degree merely adherent to the hair but appear to be integral components.

TABLE I

Effect of Washing with Various Solvents on Vitamin Content of Hair of Normal Albino Rats

	Vitamins per gm dry hair		
	Riboflavin	Pantothenic acid	Nicotinic acid
	γ	γ	γ
Hair not washed before grinding	3 7	7 8	14 1
Ground hair washed with ether	3.5	6 2	13 8
" " " " alcohol; alcohol solution analyzed*	0.6	0.0	0 0
Hair washed with water, alcohol, and ether before grinding •	3.0	7.2	10.5

* The alcohol was evaporated and the residue dissolved in 10 cc. of distilled water.

Washing the ground hair with alcohol or ether to remove fatty acids also does not appear to be essential.

That the hot water extraction of vitamins from the ground hair was complete was indicated by the failure to find any further vitamin in the extracted hair residue on further extraction. In one series of tests the residue was extracted with hydrogen sulfide. No further pantothenic acid was extracted by this procedure which had been previously shown not to be destructive of pantothenic acid (Fig. 1).

Further treatment of the residue with trypsin at pH 8 also gave no additional pantothenic acid or nicotinic acid. Another check consisted in determining nicotinic acid directly in the ground hair by acid hydrolysis. The same values were obtained as with the hot water extract. These results are not surprising, considering the fact that practically all of the hair is rendered water-soluble by the grinding process.

Regarding the possible destruction of the vitamins by the grinding process, it was found that when a given hair was ground first for 3 days and then a portion ground further for 3 days longer, similar results were ob-
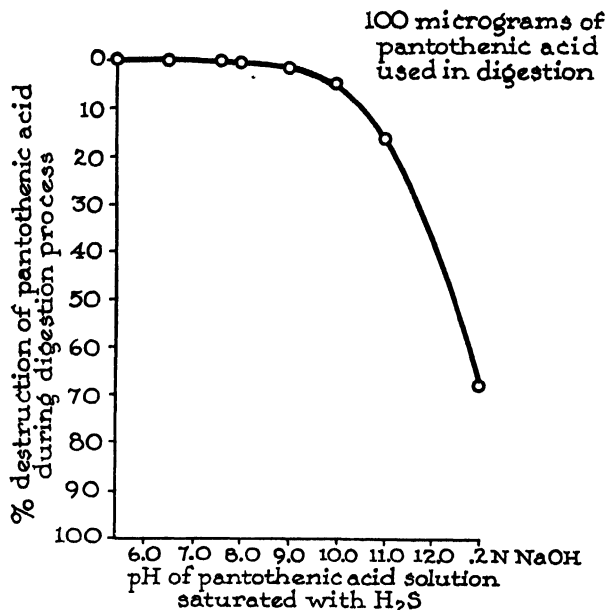
FIG 1   Extraction of vitamins from hair

TABLE II

Stability of Vitamins to Grinding

	Water-soluble vitamin content of dry ground hair		
	Riboflavin	Pathothenic acid	Nicotinic acid
	γ per gm.	γ per gm.	γ per gm
Normal albino rat hair ground 3 days.....	4.5	12.0	14.0
" " " " " 6 "	4.0	13.0	15.8
Human black hair ground 3 days...... .. .	0.2	0.5	1.8
" " " " 6 "	0.3	0.5	3.0

tained, indicating no destruction of vitamins in the 3 additional days of grinding (Table II).

The fact that ground hair of the normal albino rat shows ratios in the concentration of the vitamins to each other, similar to ratios found in other rat tissues, also suggests that no great destruction of any one vitamin was

likely to have occurred (7).   If destruction occurred, it would be expected that the very stable nicotinic acid would show relatively high results but this is not the case.

Table III presents some results on normal albino rat and human hair.

In further support of the microbiological analyses it may be of interest to cite an experiment in which ground hair was fed to rats deficient in the

TABLE III

Results of Method As Applied to Rat and Human Hair

Type of hair	Hair* extracted by various processes from ground material	Total loss on ignition after grinding	Water-soluble vitamins per gm on dry basis				Nicotinic acid in hydrolyzed ground hair†
			Ribo-flavin	Panto-thenic acid	Nico-tinic acid	Ino-sitol	
	per cent	*per cent*	γ	γ	γ	γ	γ *per gm. dry hair*
Young normal albino rat	93.0	95 5	4.5	10.0	15.0	180	17.0
" " " " .		87.0	2.8	13.0	17.0	210	18.5
" " " "	46.0	48.2	3.1	5.4	19.3	272	22.8
" " " "	59 0	64.0	4.0	9 5	21.0	215	19.5
" " " "	57.0	61.3	3.8	7.0	15.6	190	15.8
Old normal albino rat .	58 5	59.3	2.5	41 0	15.0	180	18.0
" " " " . .		58.0	2.9	8.5	12.0	150	14.5
Young normal human; fiery red hair; male; age 13 yrs.	35 0	38.3	1.2	1.2	5.7	361	7.5
Gray-haired man; full head of hair; age 62 yrs	40 0	43 7	0.9	0.5	4.6	28	5.2
Blonde man; full head of hair; age 30 yrs. .	41 0	40.8	1.3	0.5	10.5	200	10.0
Brown-black hair from middle aged man; full head of hair	50.0	55 4	1.5	0 3	6.9	250	9.1

* That is by water extraction, H_2S digestion with borate buffer at pH 10, and H_2S and trypsin digestion

† Ground hair hydrolyzed with 7 N HCl for 8 hours, neutralized, filtered, concentrated, and the neutral concentrate analyzed for nicotinic acid.

water-soluble vitamins and definite improvement in growth was observed. Such an experiment is illustrated in Fig. 2.

Influence of Diet on Water-Soluble Vitamin Content of Rat and Human Hair

Many studies have been carried out in an attempt to relate the vitamins of the diet to alopecia, achromotrichia, and other disturbances of hair growth.   No studies, however, appear to have been reported on the vitamin content of hair as influenced by diet.   In this study analyses were made for nicotinic acid, inositol, pantothenic acid, and riboflavin in the hair of rats on different diets.   Some observations on human hair are also included.
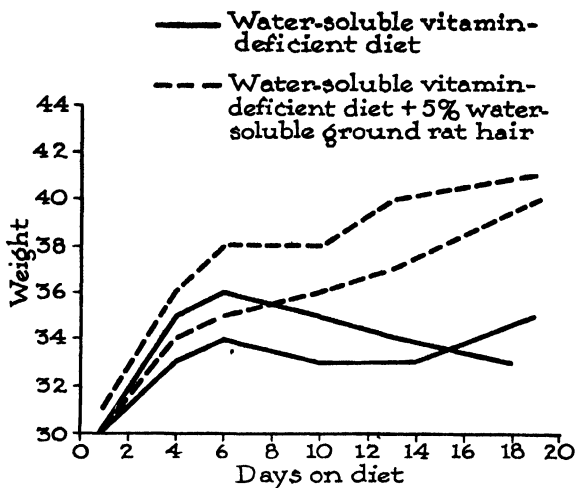
FIG. 2. Effect of feeding ground hair to rats deficient in the water-soluble vitamins

TABLE IV

Basal Diets

Constituents in diet	Amount of material in diet			
	Diet A	Diet B	Diet C	Diet D
	per cent	*per cent*	*per cent*	*per cent*
Vitamin-free alcohol-extracted casein	21	21	12	21
Corn-starch, "	66			
Dextrose......		66	83	66
Corn oil (hot water-extracted)		8.7	0.8	8.7
Butter fat (prepared)....... . .	8.5			
Salt mixture, U. S. P. XI, No. 2 ..	4.3	4.0	4.0	4.0
Vitamin A and D concentrate (Natola)	0.2	0.2	0.2	0.2
" E concentrate (Viobin)		0.1		0.1
			γ per gm.	*γ per gm*
Thiamine hydrochloride...			65	16
Riboflavin.			65	31
Calcium pantothenate				48
Pyridoxine hydrochloride			130	16
Choline hydrochloride			130	128
Nicotinic acid			130	64
Inositol.....			.	128
Biotin (crystalline methyl ester)..........				0.22

Methods—Young albino rats were kept for 30 to 40 days on one of a series of diets (see Table IV). Diet A was a diet low in all water-soluble

vitamins and contained extracted casein and corn-starch, butter fat, salt mixture, and vitamins A and D.   Diet B contained dextrose instead of starch and corn oil as the fat.   Diet C resembled Diet B but adequate

TABLE V

Water-Soluble Vitamin Content of Hair from Rats* on Various Diets

Diet	Water-soluble vitamins per gm hair on dry basis			
	Riboflavin	Panto-thenic acid	Nicotinic acid	Inositol
	γ	γ	γ	γ
A . .	0.8	0.2	8.5	
" ...	0.5	1.0	8.5	230
"...	0.4	0.7	7.6	212
" + 100 γ Ca pantothenate per gm. diet	1.0	8.5	17.0	270
" + 100 " " " " " " .	0.9	12.5	15.3	253
" + 100 " " " " " "		11.9	16.7	
" + 100 " pyridoxine per gm. diet.	3.0	2.0	4.5	60
" + 100 " " " " "	3.8	1.5	5 8	58
" + 100 " " " " "	3.7	2.9	5.1	47
" + 1% sulfaguanidine	2.2	3.0	1.2	98
" + 1% "	1.8	2.3	4.0	80
B .	2.4	0.2	9.3	191
"	1.8	0.6	7.1	199
"	2.0	0.8	7.0	243
" + 100 γ pyridoxine per gm. diet	3.9	0.6	11.5	45
" + 100 " " " " "	2.8	1.4	12.7	140
" + 200 " choline per gm. diet	2.1	1.2	19.1	30
" + 200 " " " " "	1.8	1.5	19.8	49
C	4.2	0.3	19.4	303
"	3.2	1.1	23 6	250
"	3.0	0.8	20 6	190
"	3.8	1.1	19.8	270
"	3.5	7.2	18.5	250
D	4.2	10.0	22.0	210
" .	4.0	9.2	18.0	200
" .	3.2	8.5	17.5	190
Stock diet .	4.5	10.0	15.0	180
" "	4.0	9.5	21.0	215

* Animals on Diet A or Diet A plus 100 γ of pyridoxine were fed for 40 days before hair samples were taken.   All other animals were fed for 30 days before hair samples were taken.   Pyridoxine and choline were given as the hydrochloride.

amounts of certain water-soluble vitamins were added, and it contained less casein as well as more dextrose.   In Diet D, all the well defined B complex factors were added.

Discussion—Table V shows the analytical results obtained on rat hair.

They show that the vitamins exist in somewhat the same ratio to each other as in most other rat tissues when an adequate diet is fed.

Some quantitative decrease from the normal was noted for each of the vitamins studied in certain cases. Thus pantothenic acid values fell to about one-tenth the normal values on the deficient Diet B. Similar low values were found on the deficient Diet A and addition of pantothenic acid to this diet brought the values back to normal. With reference to inositol, the most striking results were those showing that addition of choline or pyridoxine to diets deficient in the water-soluble vitamins markedly lowered the inositol content of the hair. The explanation of this result is not yet clear but it correlates significantly with low hair nitrogen to sulfur ratios on these diets,[1] as well as a tendency to alopecia in rats in these groups (9).

TABLE VI

Water-Soluble Vitamin Content of Human Hair from Men with Varying Degrees of Alopecia

Hair source and description	Water-soluble vitamins per gm. dry hair			
	Riboflavin	Panto-thenic acid	Nicotinic acid	Inositol
	γ	γ	γ	γ
Partially bald, age 30 yrs	0	0.7	2.5	
" " " 55 " . .	0.2	0.6	1.8	95
" " " 60 "	0 1	0.8	1 8	70
Partial alopecia areata in young man ingest-ing vitamin B complex tablets	0	1.5	6.5	95

Table VI gives some data for human hair. Tables II, III, and VI show that human hair contains less water-soluble vitamins than rat hair with the possible exception of inositol Many more analyses of human hair will be required to draw definite conclusions as to any relation of hair vitamin content to grayness and baldness. Our results do not indicate any definite alterations in this respect except perhaps in the case of inositol. Lower than average values were found in certain cases of baldness.

SUMMARY

A procedure is described for the determination of water-soluble vitamins in hair involving the grinding of the hair in a ball mill, extraction with hot water, and application of microbiological methods.

Data are presented illustrating the application of this procedure to the determination of pantothenic acid, riboflavin, nicotinic acid, and inositol in rat and human hair.

[1] Data to be published.

Riboflavin, nicotinic acid, pantothenic acid, and inositol are found in normal rat and human hair in about the same ratio to each other as in other tissues. Human hair has a lower content of the vitamins than rat hair. The vitamin content of rat hair may be influenced by diet. Low values for pantothenic acid were found on the vitamin-deficient diets and addition of pantothenic acid to such diets resulted in normal values for this vitamin. There is some evidence that choline or pyridoxine may lower the inositol content of hair in the rat. Low inositol values were observed in human hair from certain cases of baldness. Further studies are necessary to establish the significance of these findings.

BIBLIOGRAPHY

1 Goddard, D. R , and Michaelis, L , *J Biol Chem* , **106,** 605 (1934)
2. Routh, J. I., *J Biol Chem* , **135,** 175 (1940)
3. Routh, J I , and Lewis, H B , *J Biol Chem.*, **124,** 725 (1938)
4 Snell, E. E , and Wright, L. D , *J Biol. Chem* , **139,** 675 (1941)
5 Strong, F. M , *Ind and Eng Chem* , *Anal Ed* , **14,** 909 (1942).
6 Silber, R H , and Unna, K , *J Biol Chem* , **142,** 623 (1942)
7. Williams, R J , and Stout, A K , *Univ Texas Pub* , *No 4137,* 14 (1941)
8. Williams, R J , *Univ Texas Pub* , *No 4237,* 10 (1942)
9 Woolley, D W , *Science,* **384,** 92 (1940); *J Biol Chem.*, **139,** 29 (1941)

COLORIMETRIC ESTIMATION OF PHENYLALANINE IN SOME BIOLOGICAL PRODUCTS*

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The colorimetric estimation of phenylalanine in protein hydrolysates as described by Kapeller-Adler (1) is based on two fundamental principles: complete suppression of the interference from tyrosine and histidine, and the quantitative nitration and reduction of phenylalanine to the violet-colored ammonium salt of diaci-*o*-dinitrobenzoïc acid. Although this procedure has points of superiority over other available techniques, in practice it presents several technical difficulties which tend seriously to affect the accuracy of the final result. These difficulties arise chiefly from (*a*) the use of phosphotungstic acid for the removal of histidine, (*b*) the evaporation of the sample to dryness in preparation for nitration, and (*c*) the use of concentrated ammonium hydroxide for the reduction process. It is the purpose of this report to present experiments which have not only overcome these obstacles to satisfactory results with the original Kapeller-Adler method, but also afford greater convenience and rapidity of operation.

The removal of histidine and tyrosine, which was recommended by Kapeller-Adler, has been reported by Block *et al.* (2) as unnecessary if the color reading is made with Filter 56. In our hands, this simplification has not been found satisfactory when data of high accuracy are desired. It appears from our study that the yellow color which results from tyrosine and histidine introduces an error which is roughly proportionate to the amounts of these amino acids present in the protein. The error is particularly great when the amount of phenylalanine in the protein is small in relation to the quantity of tyrosine and histidine. Inasmuch as the destruction of tyrosine by means of permanganate offers no difficulties, the accuracy of the data in part becomes contingent on finding adequate means for the removal of histidine. The successful use of phosphotungstic acid for this purpose depends entirely upon accurately estimating the amount of this reagent required for the sample in hand. An excess leads to low phenylalanine values due to (*a*) precipitation of phenylalanine as the phosphotungstate (3), and (*b*) interference of reagent with color development.

On the other hand, an insufficient quantity of the reagent results in high phenylalanine values, owing to a failure to precipitate all of the histidine. It is obvious that considerable trial and error experimentation is necessary before satisfactory results can be obtained. These circumstances led us to attempt the removal of histidine by adsorption on permutit (4). This device has proved most satisfactory, since, as will be demonstrated, the histidine is quantitatively adsorbed and the phenylalanine is not adsorbed at all. Moreover, this procedure obviates the delay in the determination which is requisite for achieving complete precipitation of histidine as the phosphotungstate.

A second obstacle to consistent results in the original procedure arises from the manner in which the sample is evaporated to dryness before nitration. Evaporation on the steam bath as recommended by Kapeller-Adler was found to result in destruction of phenylalanine owing to over-heating of the peripheral surfaces. The errors from this source were found to be promptly remedied when the samples were evaporated in a 120° oven, a technique which reduces the time for the operation to about half that otherwise required.

The use of concentrated ammonium hydroxide for the development of color was found objectionable for three reasons: (a) danger to the operator in attempting to measure out necessary amounts, (b) possible contamination of samples for nitrogen analysis from escaping ammonia, and (c) diurnal variations in the concentration of ammonia in the reagent which may affect the color intensity. All three difficulties were overcome by substituting ammonium sulfate followed by 20 per cent sodium hydroxide.

EXPERIMENTAL

Reagents[1]—

Permutit. A 60 mesh product was used and was activated as described by Whitehorn (4).

Nitration mixture. 10 gm. of potassium nitrate dissolved in 100 cc. of concentrated sulfuric acid by shaking the mixture at room temperature.

Acid permanganate solution. 5 gm. of potassium permanganate dissolved in 100 cc. of 10 per cent sulfuric acid solution.

Hydroxylamine-ammonium sulfate mixture. 15 gm. of hydroxylamine hydrochloride and 20 gm. of ammonium sulfate dissolved in 100 cc. of distilled water.

Sodium hydroxide. 10 and 20 per cent solutions.

Phenylalanine standard. 100 mg. of *dl*-phenylalanine (Merck, 8.47 per cent N) dissolved in 100 cc. of distilled water. Since quantitative transfer of this substance is difficult, it is recommended that the amino acid be weighed directly in the volumetric flask.

[1] Merck reagent quality chemicals were used throughout

l(−)-Tyrosine (Merck) and l(+)-histidine monohydrochloride monohydrate (Merck) solutions were prepared in suitable concentrations. When reference is made to histidine, the correction for the monohydrochloride monohydrate has been made.

Biological Products—

Commercial proteins. The preparations examined include casein (Harris), lactalbumin (Harris), amigen (Mead Johnson and Company), gelatin, U. S. P. (Eimer and Amend), and fibrin (Wilson Laboratories). The analyses were performed without further refinement of the product.

Human hemoglobin. This protein was prepared from human red blood cells by the method of Zinoffsky (5). 2 liters of red cells yielded 143 gm. of crystalline hemoglobin which contained 13.96 per cent nitrogen, uncorrected for moisture and ash.

Human hair. Heterogeneous black hair obtained from barber shop clippings was used for this analysis. The uncorrected nitrogen content by micro-Kjeldahl determination is 10.15 per cent.

Stock Rats—Three normal immature animals weighing 82.74, 81.5, and 84 gm. respectively from a hybrid albino and hooded Norwegian colony were employed. The abdominal cavity was opened and the contents of the gastrointestinal tract were discarded. The entire carcasses were then submitted to acid hydrolysis. The results of these analyses are reported as an average figure. The nitrogen content of the moist tissue was 3.12 per cent.

Preparation of Hydrolysates—Constant boiling hydrochloric acid (approximately 20 per cent) was used throughout as the hydrolyzing agent. A ratio of 5 cc. of this reagent for each gm. of product was found suitable for effecting complete hydrolysis on refluxing the mixtures for 22 to 24 hours. The hydrolyses were performed in an all-glass (Pyrex) apparatus. Since the hydrolysates were required for other analyses, larger quantities (25 to 50 gm.) of material were used than were actually required for the phenylalanine estimation. For this purpose alone, the equivalent of 25 to 50 mg. of protein nitrogen is ample.

The excess of hydrochloric acid was removed *in vacuo* by three successive concentrations and additions of water. The final product was quantitatively transferred to a volumetric flask of suitable size, the volume adjusted, and aliquots removed for nitrogen determination by the micro-Kjeldahl procedure (6). The removal of humin is not necessary, as this is filtered out by the subsequent passage through permutit. The fat present in the rat hydrolysates was removed by a preliminary gravity filtration through wet fluted Whatman paper No. 12.

Analytical Procedure—A sample of the hydrolysate containing 25 to 50 mg. of nitrogen is transferred to a 50 cc. volumetric flask, diluted with water to about 25 cc., the reaction is adjusted to pH 4 (hydrion paper) with

10 per cent sodium hydroxide, and the mixture made to volume. This solution is passed at the rate of 1 drop per second through a column of 10 gm. of permutit contained in a 150 mm. calcium chloride tube plugged with coarse glass wool and fitted with a short piece of rubber tubing and a pinch-cock to regulate the flow. If the proportion of 25 to 50 mg. of total nitrogen to 10 gm. of properly activated permutit is maintained, the solution will be found negative to Knoop's test for histidine. A positive test points to poorly activated permutit. Duplicate 3 and 5 cc. samples of the solution are transferred to 30 cc. porcelain evaporating dishes and the tyrosine is destroyed by the dropwise addition of an excess of acid permanganate solution. The evaporating dishes are placed in an oven at 120°. Duplicate 1 and 2 cc. samples of the phenylalanine standard also in 30 cc. evaporating dishes are simultaneously evaporated to dryness in
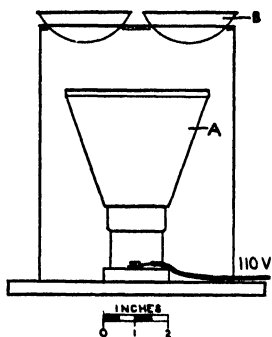
Fig 1. Diagram of hot air bath   A, 150 watt Mazda projector flood lamp. B, 30 cc evaporating dishes.

the oven. Care should be taken to remove the samples from the oven soon after they become dry, as prolonged heating (18 to 24 hours) will result in the destruction of phenylalanine. To each of the residues of the samples and standard are added 2 cc. of nitration mixture in such a manner that the residue is washed down from the sides. Nitration is completed by heating the samples on a hot air bath shown in Fig. 1 for 30 minutes. Heating on the steam bath was found to inhibit complete nitration owing to escaping steam. The contents of the evaporating dishes are transferred to 50 cc. volumetric flasks with the aid of a small funnel and a minimum amount (5 to 10 cc.) of distilled water; 5 cc. of hydroxylamine-ammonium sulfate reagent are added to each sample and, after 5 minutes, 20 cc. of 20 per cent NaOH are added slowly from a dispensing burette. The volume of each test solution is adjusted to the 50 cc. mark with distilled water and the flasks are cooled in an ice bath for 10 minutes. The resulting

solutions are read in the Klett-Summerson photoelectric colorimeter with Filter S-54. The color intensity of these reaction mixtures has been found to remain constant for about 1 hour after the initial 10 minute period of development.

Results

In order to determine the quantitative effect of the modifications made in the conditions of nitration and reduction reactions, suitable aliquots of the phenylalanine standard were submitted to the manipulations previously described. The results of these experiments are shown in Fig. 2. The linear relationship of the color intensity to the amount of phenylalanine is
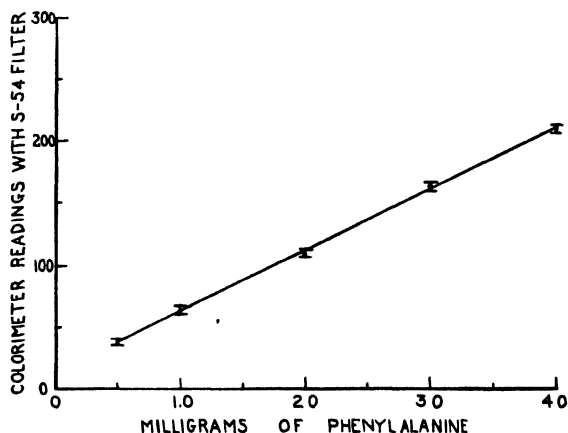
FIG. 2. Relation of color intensity to amount of phenylalanine. Each point represents the average value of ten determinations. The bars above and below each point indicate the deviation range of the readings

interpreted as evidence of the adequacy of the modifications and of the validity of Beer's law for the color reaction.

When samples of the phenylalanine standard were dried on the steam bath instead of the oven, colorimeter readings ranging from 40 to 55 and from 75 to 90 were obtained for 1 and 2 mg. of phenylalanine respectively. A comparison of these values with those obtained for similar amounts of phenylalanine by the drying oven technique (Fig. 2) indicates that a considerable loss of phenylalanine is incurred when drying is done on the steam bath.

In order to study the color interference which results from the presence of histidine and tyrosine, determinations were made upon the three amino acids singly and in mixture. The photoelectric readings obtained with

various filters and their equivalents as phenylalanine are recorded in Table I. It is evident that none of these color filters permits elimination of the procedures for the removal of histidine and tyrosine when data of high accuracy are needed.

The effect of the removal of histidine by adsorption on permutit and the oxidative elimination of tyrosine by the action of permanganate on the

TABLE I

Colorimetric Behavior of Tyrosine and Histidine in Kapeller-Adler Reaction and Its Interference to Phenylalanine Determination

Amino acids	Photoelectric readings and equivalent amino acid as phenylalanine*							
	Filter 40	Filter 42	Filter 47	Filter 52	Filter 54	Filter 56	Filter 60	Filter 66
dl-Phenylalanine, 1 mg.	121	151	65	60	64	57	40	11
	1.00	1 00	1.00	1.00	1.00	1.00	1.00	1.00
l(−)-Tyrosine, 1 mg	198	296	40	13	15	9	5	0
	1.64	1.96	0.62	0 22	0.22	0.16	0.12	0
l(+)-Histidine, 1 mg	80	65	25	12	11	5	8	2
	0 66	0.43	0 38	0 20	0.17	0.08	0.20	0.18
dl-Phenylalanine, 1 mg., l(−)-tyrosine, 1 mg., l(+)-histidine, 1 mg	240	480	92	78	86	65	45	12
	1.98	3.18	1.42	1.30	1.34	1.14	1.12	1.10

* The first reading for the respective amino acids represents the photoelectric reading; the second the equivalent mg of amino acid measured as phenylalanine.

TABLE II

Recovery of Phenylalanine Added to Amino Acid Mixtures

Amino acid mixture	Phenylalanine in sample	dl-Phenylalanine added*	Total phenylalanine found	Recovery of added phenylalanine
	mg	*mg*	*mg*	*per cent*
l(+)-Histidine, 0.5 mg., l(−)-tyrosine, 0.5 mg.	0.0	1.0	1.02	102.0
Casein hydrolysate, 5.53 mg. N	1.93	1.0	2.93	100 0
Gelatin " 11 76 " "	1.80	1.0	2.82	102.2

* Phenylalanine added before treatment of composite sample with permutit

phenylalanine added to a mixture of tyrosine and histidine and to protein hydrolysates was also investigated. Representative data are given in Table II. The quantitative recovery of added phenylalanine points to the adequacy of these steps for analytical purposes.

The results of the phenylalanine analyses of the biological products are reported in Table III. The inaccuracy introduced in the phenylalanine

values of casein, human hemoglobin, and human hair by the failure to remove tyrosine and histidine is demonstrated by data recorded in Table IV.

Comment

Although the modifications of the original Kapeller-Adler procedure for the estimation of phenylalanine suggested by this investigation have been found to be time-saving, they also improve the accuracy of the determina-

TABLE III

Phenylalanine Content of Some Biological Substances

Biological product	Nitrogen content	Phenylalanine N of total N	Phenylalanine per 100 gm. product*
	per cent	*per cent*	*gm.*
Casein	13.62	2.98	4.77
Amigen	11.80	2.56	3.76
Lactalbumin	11.70	2.08	2.88
Gelatin	14.30	1.30	2.24
Fibrin	15.10	2.75	4.92
Human hemoglobin	13.96	5.06	7.84
" hair	10.15	0.11	0.13
Stock rats	3.12	2.57	0.94

* Not corrected for moisture or ash content

TABLE IV

Effect of Presence of Tyrosine and Histidine on Phenylalanine Values

Protein	Phenylalanine N of total N	
	Author's method	Histidine and tyrosine present; read with Filter 56
	per cent	*per cent*
Casein	2.98	4.17
Human hemoglobin	5.06	4.48
" hair	0.11	0.16

tion. On suitable standardization of each step it is possible for a trained technician to perform a complete analysis in 3 to 4 hours with duplicate checks of better than 1 per cent. When the original method was used, the results of these assays were delayed 24 to 48 hours in order to achieve complete precipitation of histidine phosphotungstate. Moreover, possibly due to variations in the solubility of this salt, the results were erratic. The elimination of the use of concentrated ammonium hydroxide has been found to afford greater safety to the operator.

SUMMARY

Three improvements have been made in the original procedure of Kapeller-Adler for the colorimetric estimation of phenylalanine which effect a saving of time, and obtain greater accuracy and convenience of operation. Of the seven biological products analyzed by the modified method, crystalline human hemoglobin was found to have the highest phenylalanine content.

BIBLIOGRAPHY

1. Kapeller-Adler, R , *Biochem Z.*, **252**, 185 (1932).
2 Jervis, G. A , Block, R J., Bolling, D., and Kanze, E , *J. Biol Chem* , **134**, 105 (1940). Block, R. J., Jervis, G A , Bolling, D , and Webb, M , *J. Biol. Chem.*, **134**, 567 (1940).
3. Schulze, E., and Winterstein, E., *Z. physiol. Chem* , **35**, 210 (1902)
4. Whitehorn, J. C , *J Biol. Chem* , **56**, 751 (1923)
5. Zinoffsky, C , *Z. physiol. Chem.*, **10**, 16 (1886).
6. Meeker, E. W , and Wagner, E. C , *Ind and Eng Chem* , **5**, 396 (1933)

THE INTERMEDIARY METABOLISM OF TRYPTOPHANE IN PYRIDOXINE-DEFICIENT RATS

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It has been shown that the xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic acid) found in the urine of pyridoxine-deficient rats originates from dietary $l(-)$-tryptophane (1). Since it is known that many compounds closely related to tryptophane promote growth in the rat (2–8) and give rise to kynurenine and kynurenic acid in the urine (2, 9–15), it seemed of interest to investigate some of these compounds as possible precursors of xanthurenic acid in pyridoxine-deficient rats. Accordingly, studies have been made on the ability of pyridoxine-deficient rats to convert into xanthurenic acid the following: $d(+)$-tryptophane, indole-3-pyruvic acid, indole-3-lactic acid, indole-3-acetic acid, indole-3-propionic acid, abrine (N-methyl-$l(-)$-tryptophane), kynurenine, kynurenic acid, and indole + serine (16).

EXPERIMENTAL

Commercial preparations of $l(-)$-tryptophane, indole, dl-serine, indole-3-propionic acid, and indole-3-acetic acid were used. The sample of $d(+)$-tryptophane was given us by Dr. C. P. Berg, Iowa State University. The sample of abrine was kindly supplied by W. M. Cahill, Wayne University. Indole-3-pyruvic acid, kynurenine, and kynurenic acid were prepared as follows:

Indole-3-pyruvic Acid—This acid was prepared from indole aldehyde as described by Ellinger and Matsuoka (11), the indole aldehyde having been prepared from indole by the method of Boyd and Robson (17). The final product was a buff-colored powder with an indefinite melting point. Preparation of the p-nitrophenylhydrazone yielded a crystalline product with a melting point in agreement with that reported by Ellinger and Matsuoka (11) and by Berg, Rose, and Marvel (5), and elementary analysis gave the following results.

$C_{11}H_9NO_3$ Calculated. C 65.01, H 4.47, N 6.85
　　　　　　　Found.　　" 65.69, " 4.61, " 7.12

Kynurenic Acid—The sodium salt of diethyloxalacetate was prepared by allowing diethyloxalate (prepared by the method of Clarke and Davis (18)) to react with sodium and ethyl acetate in anhydrous ether (19). 20 gm. of sodium diethyloxalate were allowed to react with 10 gm. of aniline hydrochloride in ethyl alcohol overnight at room temperature (20). The reaction mixture was poured into about 1 liter of water and extracted with ether. The ether solution containing the anil was evaporated after drying over anhydrous Na_2SO_4, leaving a viscous yellow oil. This oil was then heated in an open tube held in an oil bath at 190–200° for 1 hour. Upon cooling, the oil readily crystallized. This product consisted of two fractions, ethyl kynurenate, readily soluble in ethyl alcohol, and a second fraction, somewhat soluble in hot alcohol and insoluble in cold alcohol. The product was therefore entirely dissolved in hot ethyl alcohol and allowed to stand overnight in the cold room. The crystals were then filtered off, the filtrate evaporated to dryness, and the residue refluxed with 10 per cent NaOH for 1 hour. The alkaline solution was decolorized with norit and upon acidification gave a bulky white precipitate which crystallized as needles from aqueous acetic acid. The melting point of the hydrate was 287–288°. When boiled with acetic anhydride a red color developed (21) and elementary analysis gave the following results.

$C_{10}H_9NO_3$. Calculated. C 63.15, H 4.24, N 7.37
            Found.   " 63.23, " 4.14, " 6.88

Kynurenine Sulfate—*l*-Kynurenine sulfate was prepared from *l*-tryptophane by the action of an unidentified *Bacillus* sp. according to the procedure described by Tatum and Haagen-Smit (22).

Indole-3-lactic Acid—Indole-3-lactic acid was prepared by the use of *Oidium lactis* by the method of Ehrlich and Jacobsen (23). The product was purified by reprecipitation from ether with petroleum ether. The properties of the final product were similar to those reported by Ehrlich and Jacobsen (23). A micro-Dumas nitrogen determination gave the following results

$C_{11}H_{11}O_2N$. Calculated, N 6.83; found, 7.30

Diets, Care of Rats, and Methods of Testing—At weaning rats were put on a pyridoxine-deficient diet consisting of casein 27 parts, dextrose 58, Crisco 5, lard 5, Sure's Salt Mixture 1 (24) 4, "sardilene" oil[1] 1. To each kilo of diet were added thiamine hydrochloride[2] 10 mg., riboflavin[2] 10 mg., Ca pantothenate[2] 40 mg., choline 500 mg., nicotinic acid 100 mg., *p*-aminobenzoic acid 600 mg., inositol 1 gm., biotin[2] 25 γ, 1-acetoxy-2-methyl-4-naphthyl sodium

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[1] Sardilene, a commercial fish oil containing 400 units of vitamin D (largely vitamin D_3) and 3000 units of vitamin A per gm.

[2] Kindly supplied by Merck and Company, Inc , Rahway, New Jersey.

phosphate 20 γ, and "vitamin E,"[3] distillate equivalent to 10 mg. of tocopherols.

Previous experiments have shown that from $2\frac{1}{2}$ to 3 weeks on this diet are required for a rat to become sufficiently depleted in pyridoxine to excrete xanthurenic acid. A positive ferric ammonium sulfate test (the appearance of a green color when iron salts are added to the urine) (1) was taken as an indication of xanthurenic acid in the urine, and thus of pyridoxine deficiency.

Two deficient animals were placed in a metabolism cage constructed with glass rod flooring and glass funnel so that the urine was kept from contact with metal at all times. Feces were prevented from falling into the urine by a Witte plate placed in the funnel.

The rats were then fed a pyridoxine- and tryptophane-deficient diet identical with that previously described except that the casein was replaced by an equal amount of acid-hydrolyzed casein plus 0.3 per cent cystine. Water was given *ad libitum*. Under these conditions, xanthurenic acid (as evidenced by the ferric ammonium sulfate test) disappears rapidly from the urine. Accordingly urine samples were tested with ferric ammonium sulfate at 2 hour intervals until the green color no longer formed (about 3 or 4 hours in contrast to the previously reported time, 6 to 12 hours (1)). This was considered an indication that the animals were deficient in tryptophane as well as in pyridoxine. 4 to 6 hours were allowed to elapse before the substance to be tested was fed.

The compounds investigated were fed in amounts equivalent to 30 mg. of tryptophane. Each rat was fed the calculated amount by stomach tube and the urine was examined hourly for the presence of xanthurenic acid. A similar amount was given after 6 hours and the urine similarly tested. If the results were negative, the animals were given 30 mg. of $l(-)$-tryptophane and in every instance there followed positive tests for xanthurenic acid in from 2 to 4 hours.

All substances were fed again after the rats had received intraperitoneally an excess of pyridoxine (100 γ), since previous work (1) showed that 20 γ were sufficient to cause the immediate disappearance of xanthurenic acid from the urine of a pyridoxine-deficient rat when $l(-)$-tryptophane was the precursor.

Duplicate tests were made with all substances. Of all the compounds fed, only $l(-)$-tryptophane and kynurenine were converted to xanthurenic acid by the pyridoxine-deficient rat. When pyridoxine was fed in addition, no xanthurenic acid was produced.

DISCUSSION

Since only $l(-)$-tryptophane and kynurenine of the compounds fed yielded xanthurenic acid in the pyridoxine-deficient rat, this work can be considered supporting evidence with the pyridoxine-deficient rat for the ideas advanced by Musajo and Chiancone (25) that kynurenine is an intermediary product in the conversion of $l(-)$-tryptophane to xanthurenic acid. It should be pointed out that of the remaining compounds $d(+)$-tryptophane (2, 3), indole-3-pyruvic acid (4, 5), indole-3-lactic acid (26), and abrine (27, 28) can replace $l(-)$-tryptophane for growth. Apparently at least in the pyridoxine-deficient rat the metabolic degradation of $d(+)$-tryptophane, indole-3-pyruvic acid, indole-3-lactic acid, and abrine differs from that of $l(-)$-tryptophane. The inability of the pyridoxine-deficient rat to convert kynurenic acid to xanthurenic acid indicates that the conversion of kynurenine to kynurenic acid is not reversible.

As would be expected, xanthurenic acid was excreted unchanged in the pyridoxine-deficient rat, but when pyridoxine was fed, xanthurenic acid could not be recovered from the rat urine. The fate of such xanthurenic acid under these conditions is at present unknown.

SUMMARY

1. Kynurenine and $l(-)$-tryptophane yield xanthurenic acid in the pyridoxine-deficient rat.

2. Xanthurenic acid passes through the pyridoxine-deficient rat unchanged, but cannot be recovered from the urine of pyridoxine-fed rats.

3. Indole-3-pyruvic acid, $d(+)$-tryptophane, indole-3-propionic acid, indole-3-lactic acid, indole-3-acetic acid, abrine, indole + serine, and kynurenic acid do not form xanthurenic acid in pyridoxine-deficient rats.

BIBLIOGRAPHY

1. Lepkovsky, S., Roboz, E , and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943)
2. Berg, C P , *J Biol. Chem* , **104**, 373 (1934).
3. du Vigneaud, V., Sealock, R R., and Van Etten, C., *J. Biol Chem.*, **98**, 565 (1932)
4. Jackson, R. W., *J. Biol. Chem.*, **84**, 1 (1929).
5. Berg, C. P., Rose, W. C., and Marvel, C. S , *J. Biol. Chem* , **85**, 219 (1929–30)
6. Asayama, C., *Biochem. J.*, **10**, 466 (1916).
7. Jackson, R. W., and Jackson, W. T., *J. Biol. Chem.*, **96**, 697 (1932).
8. Berg, C. P., and Potgieter, M., *J. Biol. Chem.*, **94**, 661 (1931–32).
9. Matsuoka, Z., *J. Biol. Chem.*, **35**, 333 (1918).
10. Berg, C. P., *J. Biol. Chem.*, **91**, 513 (1931).
11. Ellinger, A., and Matsuoka, Z., *Z. physiol. Chem.*, **109**, 259 (1920).
12. Correll, J. T., Berg, C. P., and Cowan, D. W., *J. Biol. Chem.*, **123**, 151 (1938)
13. Homer, A., *Biochem. J.*, **12**, 290 (1918).
14. Ichihara, K., and Igoto, S., *Z. physiol. Chem.*, **243**, 256 (1936)

15. Kotake, Y., and Iwao, J., *Z. physiol. Chem.*, **195**, 139 (1931).
16. Tatum, E. L., and Bonner, D. M., *J. Biol. Chem.*, **151**, 349 (1943)
17. Boyd, W. J., and Robson, W., *Biochem. J.*, **29**, 255 (1935).
18. Clarke, H. T., and Davis, A. W., Organic syntheses, New York, coll **1**, 261 (1932).
19 Wislicenus, W., *Ann. Chem.*, **246**, 315 (1888).
20 Musajo, L., *Gazz. chim. ital*, **67**, 222 (1937); *Chem Abstr.*, **31**, 8536 (1937)
21. Kertschy, M., *Monatsh. Chem.*, **2**, 76 (1881).
22. Tatum, E L, and Haagen-Smit, A J, *J. Biol. Chem*, **140**, 575 (1941)
23. Ehrlich, F, and Jacobsen, K A, *Ber chem Ges*. **44**, 888 (1911)
24. Sure, B, *J. Nutr.*, **22**, 499 (1941)
25. Musajo, L, and Chiancone, F. M, *Boll. Soc ital biol sper*, **10**, 290 (1935), *Chem Abstr*, **29**, 6292 (1935)
26. Bauguess, L C, and Berg, C. P, *J Biol Chem*, **104**, 675 (1934)
27 Gordon, W G, and Jackson, R. W., *J Biol Chem*, **110**, 151 (1935). Gordon, W. G, *J Biol Chem.*, **129**, 309 (1939)
28 Cahill, W M., and Kotalik, G. C., *J. Nutr*, **26**, 471 (1943)

THE ACTION OF THE HUMAN SMALL INTESTINE IN ALTERING THE COMPOSITION OF PHYSIOLOGICAL SALINE*

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Considerable evidence has accumulated (1, 9, 12–14, 16) to show that in the rabbit, rat, and dog there is a certain constancy about the reactions, especially pH, of the various sections of the small intestine which have the appearance of physiological constants. Data from intubation studies permit this generalization to be applied in a general way to the human. Robinson, Luckey, and Mills (15) found that the jejunum of the normal human achieves a pH of approximately 6.6 regardless of whether acid or alkaline solutions of hyper- or hypotonic $CaCl_2$ were introduced. McGee and Hastings (8) found the reaction of human jejunal secretion to be pH 6.5 ± 0.3. As regards the remainder of the small intestine, little can be said except that it is generally agreed that both the secretion (7) and the content (4, 10) become progressively less acid as the ileocecal valve is approached.

A more exact knowledge of the regional characteristics, especially as these might alter the reaction of a simple isotonic sodium chloride solution, has now become a basic requirement fundamental to the study of intestinal absorption. The need for such information is further emphasized by the fact that the medium from which absorption studies should be made must be reasonably isotonic, distilled water alone being injurious to the intestinal mucosa (2). We have attempted to ascertain the characteristics of the various levels of the human small intestine as these are reflected by the pH and change in the chemical composition of approximately isotonic sodium chloride.

Methods

Eighteen successful intubations were performed on eighteen normal human subjects, aged 17 to 25 years, by the use of the Miller-Abbott triple lumen tube equipped with a metal olive. The interballoon space was 12 to 15 cm.; the distance from the oral end of the proximal balloon to the olive was 36 to 38 cm. The tube was taken in the morning by a fasting subject.

Fluoroscopy 1½ hours later usually revealed the proximal balloon just beyond the pylorus with the olive at or beyond the ligament of Treitz. The balloons were gently distended by introducing 25 cc. of air, and the interballoon space flushed with 40 to 50 cc. of warm saline. A measured amount of the saline (40 to 60 cc.) was then introduced and allowed to remain in the intestinal segment for 15 to 20 minutes. After this time, the first sample was withdrawn under mineral oil, with gentle suction provided by a siphon arm of 30 to 40 cm. of water. The first 10 to 15 cc. withdrawn were set aside and reintroduced after the main sample of 25 cc. had been collected. With the same precautions, another sample was taken 20 minutes later, at which time the segment was emptied completely. Thus an attempt was made to obtain a 20 and a 40 minute sample on each instillation.

A period of about 20 minutes was allowed for the apparatus to reach a new level, whereupon a new instillation of saline was made and the sampling at 20 and 40 minutes repeated. This cycle of manipulations was repeated throughout the experimental period. During the interval when the interballoon space was empty, the balloons were deflated and checked for leakage. Usually the distal balloon was reinflated to furnish a stimulus for propulsive activities.

Care was taken not to distend the balloons to the point at which the subject noted the least pain; the aim was to underdistend and lose the specimen, rather than to overdistend and thereby evoke a hypersecretion. The experiments were timed from the first instillation of saline, when the apparatus had just completed its passage from the stomach. Hence all references to time are to be interpreted as hours after the pylorus had been reached.

The subject was encouraged to take the 200 to 250 cm. of tube as early in the day as possible, thus furnishing at all times sufficient tube to permit it to pass down the intestine as rapidly as it would. The withdrawal of the samples did not always proceed smoothly. Consequently out of 170 instillations, 88 were complete in the sense that both a 20 and a 40 minute specimen large enough for all analyses was obtained for each. The final position of the apparatus was always checked by x-ray.

The saline solution was made of c.p. sodium chloride and had an average composition, among the several lots, of 150 milliequivalents per liter.

All samples were analyzed immediately for pH and total CO_2. The pH determinations were made at 30–32° with a Coleman micro chamber glass electrode and corrected for 38°. The total CO_2 was determined manometrically by the method of Van Slyke and Neill (17). Total base was determined by a modification of the benzidine method. The bicarbonate content and CO_2 tension were calculated as outlined by McGee and

Hastings (8). Chloride determinations were made on the samples from the last eight intubations by means of the Volhard titration on the alkaline ash. No attempt has been made to estimate the total amounts of the various substances, since volume recoveries were too uncertain.

Results

In three of the eighteen intubations the apparatus did not advance normally, for after 6 to 9 hours it was found still at the ligament of Treitz.
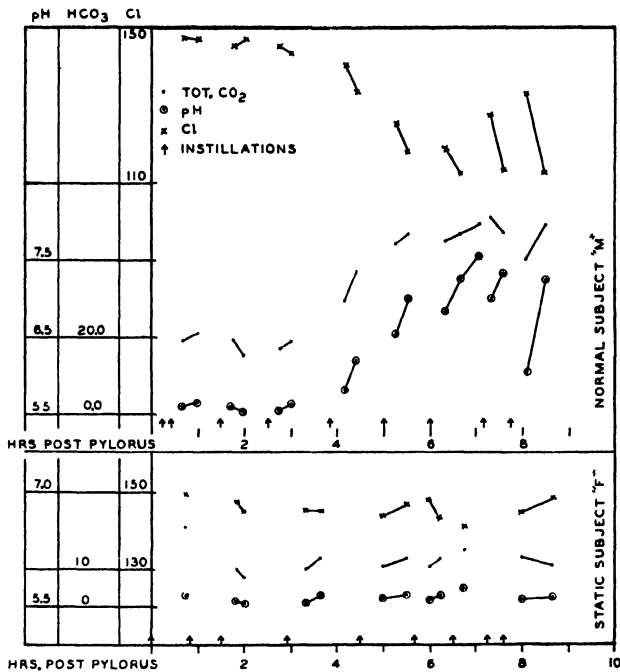
Fig 1 Changes in composition of saline instilled into the gut at various levels. The solid lines connect points in the same instillation.

These have been termed "static samples" and the data treated separately. Final fluoroscopy showed that the apparatus had passed well into the ileum or was at the ileocecal valve in nine intubations, while in six others it was located in the colon.

Fig. 1 shows the data from two typical experiments. Curve M illustrates one of the intubations on which chloride determinations were made, and the tube progressed normally to the lower ileum; Curve F is typical of the "static samples."

A most conspicuous feature about these curves is the zigzag nature of

their profiles due to the more advanced chemical change in the 40 minute compared to the 20 minute specimen for any given instillation. Nevertheless, in spite of these irregularities, in all but the "static samples" there was an obvious tendency for the total bicarbonate and pH values to increase and for the chloride values to decrease as the ileocecal valve was approached. The over-all shape of the curves was quite regular. All, in some degree, showed an initial flat section, followed, about the 4th hour, by a steep slope to the maximum bicarbonate and minimum chloride concentrations. This break may serve as a landmark for the chemical characterization and identification of a definite segment not yet recognized on other grounds.

The differences observed between any two samples on a single instillation may be the result of two factors acting in combination: (1) an absorption-secretion process which progresses with time and is more manifest in the older, 40 minute sample, *i.e.* a simple time factor, and (2) a time-distance factor, *i.e.* the attainment of more distal mucosal surfaces with differing chemical characteristics. The extent to which these two factors participate during the time of one instillation was approached statistically. A comparison of the chemical data on the 20 and 40 minute samples of 76 complete instillations shows that *time* is one important factor, especially in the cases with good motor activity. The mean values of the 76 pairs of data with respect to the various determinations, as well as the derived values for bicarbonate and carbonic acid, along with their standard errors and critical ratios are given in Table I. The pH, total CO_2, and the derived values for bicarbonate are all *significantly higher* in the 40 minute samples; the total base remains constant, while the chloride concentration is *significantly lower* in the older sample.

In contrast to these findings are those on the "static samples" in which only small differences of no significance occur in all the components examined.

The evaluation of the distance factor is more difficult because intestinal distance traversed in a given time is entirely unknown. Certainly it varies with the individual and the general region of the intestine involved and the rate is probably not uniform over short periods; hence there is small basis on which the data as a group can be rigidly examined.

The average time interval separating any 40 minute specimen from the 20 minute specimen of the next instillation was 46 minutes. Assuming a fairly uniform rate, the apparatus could have advanced as far as it had during the time the 40 minute specimen was being carried. If the distance factor was a sizable one in causing the changes produced in the 40 minute specimen, a similar increment of change should be evident in the next 20 minute sample. Examination of 63 such pairs of samples with respect

to pH showed the more distal sample to have indeed a higher pH. The pH of the 40 minute samples had a mean value of 7.165 ± 0.061, while that of the more distal 20 minute sample was 7.212 ± 0.057 (probability of 6 in 100). This would seem to indicate that the chemical characteristics of the mucosa traversed by the apparatus in 46 minutes (carrying saline during the last 20) have changed to the extent that saline exposed for 20 minutes is altered slightly more than saline exposed in the previous segment for 40 minutes.

TABLE I

Summary of Changes Occurring in 150 mM of NaCl during 20 Minute and 40 Minute Periods within Small Intestine

76 installations (15 subjects)	20 min. samples		40 min samples		Critical ratio
	Mean	Standard error	Mean	Standard error	
pH, 38°	7.108	±0.0531	7.261	±0.0555	8.1
Total CO₂, *mM*	12.09	±1.096	17.88	±1.560	8.4
HCO₃, *mM*	11.27	±1.088	17.02	±1.527	8.8
CO₂, *mM*	0.824	±0.0243	0.858	±0.0244	1.8
Total base, *m.eq. per l*	146.88	±0.441	146.85	±0.425	0.08
Total chloride,* *m eq per l*	134.33	±1.289	127.32	±1.794	5.6

12 installations (3 subjects)	Static samples				Critical ratio
pH, 38°	6.259	±0.099	6.317	±0.106	0.90
Total CO₂, *mM*	3 398	±0.535	4.044	±0.736	1.41
HCO₃, *mM*	2.131	±0.614	2.710	±0.764	1.06
CO₂, *mM*	1.305	±0.157	1.333	±0.173	0.20
Total base, *m eq per l*	153.88	±0.529	152.15	±0.634	0.02
Total chloride,† *m.eq per l*	145.15	±1.02	143.4	±1.59	0 9

* Data on forty-one installations, eight subjects.

† Data on six installations, two subjects.

The importance of the distance factor is clearly substantiated in the "static samples" in which, in the absence of propulsive motility, the time factor alone was ineffective in producing any significant changes in the saline.

These factors do not appear to influence total osmotic concentration, as indicated by the constancy of the total base in all samples at all intestinal levels. The carbonic acid concentration was likewise stable, showing but a small and insignificant tendency to fall as the lower segments were reached. (See Fig. 2 which depicts the relationships between bicarbonate, CO_2 tension, and pH in the same two subjects used for Fig. 1.) Consequently the variations in pH are due to changes in the bicarbonate ion.

This is in agreement with our previous data on dogs and those of McGee and Hastings (8) for human jejunal juice.

It is our belief that the chemical changes observed are conditioned by the presence of the saline in only the small intestine and that they cease to be manifest as soon as the colon is reached. A few samples obtained
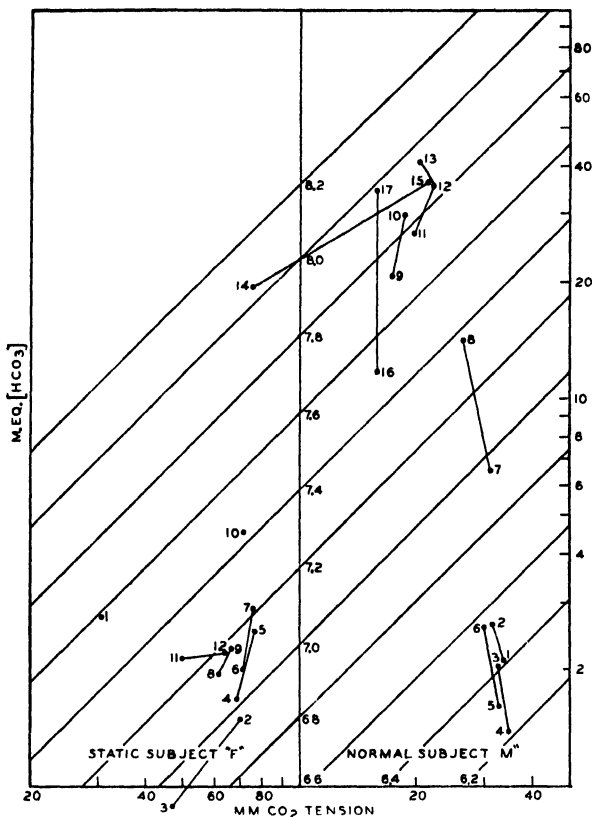
FIG. 2. Bicarbonate-CO_2 changes in relation to pH (center scale) in saline instillation in human intestines. The figures indicate the sequence of the points. The solid lines connect points in the same instillation

from the colon and some ileal samples obviously contaminated with colonic contents were distinctly less alkaline, carried less CO_2, and were higher in chloride than the definitely ileal samples from the same subject. A rather abrupt fall in the pH at the ileal-colon junction has been recorded by Graham and Emery (4) who studied the intestinal contents of freshly killed dogs.

DISCUSSION

Our data confirm the prevailing belief that the small intestine is able to adjust its contents to a more alkaline reaction as the ileocecal valve is approached. At a certain level, reached about the 4th hour, there is a marked accentuation of the gradient that makes this adjustment possible. We are unable to state directly which anatomical section is concerned, but there is indirect evidence from the literature that it is the ileum. This indirect evidence rests on the confirmed observation of Dennis and Visscher (3) and unpublished results of our own that ileal loops in dogs are far more efficient in depleting their content of chloride than jejunal loops together with the report of Ingelfinger and Abbott (6) that an intubation apparatus very similar to ours requires about 2 hours after the pylorus is reached to traverse the jejunum and 3 to 6 hours to pass through the ileum. In our cases, the marked fall in chloride, as well as the rise in total CO_2 and pH, had its beginning at the time when the apparatus might be expected to have first fully entered the ileum.

McGee and Hastings have concluded that the high carbon dioxide tension in jejunal juice is the result of a specific secretory process. If this is so, it would seem to parallel in a chemically equivalent manner a process by which chloride is absorbed or removed from the saline. From Table I, it may be verified that in 20 minutes the sodium chloride solution, originally 150 milliequivalents per liter, had gained 11.27 milliequivalents per liter of bicarbonate and lost 12.55 milliequivalents of chloride unattended by base. At the end of 40 minutes an additional 6.59 milliequivalents per liter of bicarbonate appeared, while 7.01 milliequivalents of chloride disappeared.

To explain the equivalent and reciprocal relationships of the bicarbonate and chloride ions solely on the basis of secretion would necessitate the entrance, during 40 minutes, of about 11 cc. of 146 mM sodium bicarbonate, which would thereby raise the bicarbonate ion concentration to about 25 milliequivalents per liter instead of the 17 milliequivalents actually obtained.

Although much significance cannot be attached to the volumes recovered, in 64 instillations uncomplicated by emesis or signs of obstructive hypersecretion, the average volume introduced was 54.4 ± 1.27 cc. and that recovered was 56.5 ± 2.85 cc. A consistent secretion of some 11 cc. per instillation might reasonably have been reflected in a greater difference than was obtained.

The fluid circuit theory developed by Peters and Visscher (11) to explain the chloride depletion of isotonic saline and more especially isotonic mixtures of NaCl and Na_2SO_4 by the ileum is also inadequate. This theory

assumes that a hypotonic, non-chloride secretion occurs at the same time that chloride is being actively absorbed. Such a theory demands that the osmotic activity and total base of the content show a reduction. While it will be found true that the total base for the "static samples" has an average value statistically greater than that of the others, between those samples in which a significant chloride and bicarbonate exchange did occur, there was *no change* in total base. The literature substantiates our data in regard to the amount and constancy of the total base in human succus entericus (8), in dog succus entericus (1), and in human ileal dejecta (18).

It would seem more likely that the appearance of bicarbonate is linked in some way with a definite absorption rather than dilution of the chloride. Herrin (5) has mentioned this reciprocal behavior of chloride and bicarbonate in pure succus entericus of the dog. He suggests that bicarbonate enters to compensate for chloride ion removed. He concludes that the mucosa can secrete these ions quite independently of their concentration in the blood stream.

SUMMARY

A study of the chemical composition of physiological saline introduced into the small intestine of eighteen normal human subjects permits the following generalizations:

1. In the presence of normal propulsive activity, isotonic saline exposed for 40 minutes anywhere in the small intestine has a significantly higher pH and bicarbonate content and lower chloride content than when exposed for only 20 minutes.

2. The magnitude of these chemical changes is accentuated when the ileum is concerned and attains maximum values in this region.

3. The increase in pH is the result of an increase in bicarbonate ion which appears in amounts chemically equivalent to the chloride which disappears. Total base remains constant.

4. Unless normal propulsive motility exists, no significant chemical changes occur during a period of 40 minutes as compared to a period of 20 minutes.

5. Such great variability is encountered that it is impossible to define limited regions of the small intestine in terms of discrete statistical ranges of their chemical potentialities.

BIBLIOGRAPHY

1. de Beer, E. J., Johnston, C. G., and Wilson, D. W., *J. Biol. Chem.*, **108**, 113 (1935).
2. Dennis, C., *Am. J. Physiol.*, **129**, 171 (1940).
3. Dennis, C., and Visscher, M. B , *Am. J. Physiol.*, **129**, 176 (1940).
4. Graham, W. R., and Emery, E. S., *J. Lab. and Clin. Med.*, **13**, 1097 (1928).
5. Herrin, R. C , *J. Biol. Chem.*, **108**, 547 (1935).
6. Ingelfinger, F. J., and Abbott, W. O., *Am. J. Digest. Dis.*, **7**, 468 (1940).
7. Karr, W. G., and Abbott, W. O., *J. Clin. Invest.*, **14**, 893 (1935).
8. McGee, L. C., and Hastings, A. B., *J. Biol. Chem.*, **142**, 893 (1942).
9. McRoberts, G. R., *Indian J. Med. Res.*, **16**, 545 (1928).
10. Paulson, M., *Bull. Johns Hopkins Hosp.*, **45**, 315 (1929).
11. Peters, H. C., and Visscher, M. B., *J. Cell. and Comp. Physiol.*, **12**, 51 (1938).
12. Robinson, C. S., *J. Biol. Chem.*, **108**, 403 (1935).
13. Robinson, C. S., and Duncan, C. W., *J. Biol. Chem.*, **92**, 435 (1931).
14. Robinson, C. S. and Gilliland, H. L., *Proc. Soc. Exp. Biol. and Med.*, **37**, 336 (1937).
15. Robinson, C. S., Luckey, H., and Mills, H., *J. Biol. Chem.*, **147**, 175 (1943).
16. Robinson, C. S., Stewart, D. E , and Luckey, F., *J. Biol. Chem.*, **137**, 283 (1941).
17. Van Slyke, D. D., and Neill, J. M , *J. Biol. Chem.*, **61**, 523 (1924).
18 Welch, C S., and Wakefield, E G , *Arch. Int. Med.*, **58**, 1095 (1936).

THE EFFECT OF ATABRINE ON THE OXYGEN CONSUMPTION OF TISSUES

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Atabrine has recently attained wide use as a substitute for quinine. A review of the literature indicated that atabrine interferes with the O_2 consumption of *Plasmodia* (1) without affecting the O_2 consumption of the tissues of the host (2). In view of the rather severe toxic reactions that have been observed in man and animals (3, 4), the effect of atabrine on tissue O_2 consumption was reexamined and it was found that the drug does inhibit O_2 consumption of mammalian tissues at relatively low concentrations. The point of attack is probably on the yellow enzyme systems.

Methods

Measurements of O_2 consumption were made with a Barcroft differential type of manometers fitted with side arm flasks of approximately 20 ml. capacity. The suspending medium was a phosphate-buffered (pH 7.3) physiological salt solution, as described by Dickens and Greville (5). The manometer flasks were flushed out with O_2 after being placed in the water bath at 37.4°. The tissue slices were dried at 100°, and the results calculated as c.mm. of O_2 per mg. per hour.

The *d*-amino acid oxidase was prepared as described by Krebs (6). Sheep kidneys were ground, washed with acetone, dried, and stored in a vacuum desiccator. 1.5 gm. of the dried kidney were ground with sand and 20 ml. of water, centrifuged, and the supernatant (KE) used as the enzyme preparation. In order to prepare the prosthetic group, KE was treated with 4 volumes of methanol or heated to 80° for 10 minutes. In either procedure the resulting precipitate was removed by centrifuging and the supernatant evaporated to dryness. The residue was taken up in physiological salt solution. The preparation obtained by the methanol treatment is designated KM, that obtained by heat treatment KH.

Results

The effect of atabrine on the O_2 consumption of tissue slices from organs of the rat is shown in Fig. 1. The atabrine was tipped from the side arm of the manometric flask after a control period of 45 minutes, as indicated on the time axis. The initial effect of the drug on the liver slices from a well nourished rat (Fig. 1, *A*) is a marked stimulation of O_2 consumption. This

is followed by a fall in O_2 consumption to approximately 5 per cent of the control rate.   If the rat is fasted for 24 to 48 hours immediately preceding the removal of the liver, the initial rise in O_2 consumption is greatly diminished or disappears completely (Fig. 1, B).   Such periods of fasting reduce the glycogen content of rat liver to 0.1 per cent (7).   Atabrine causes a fall in the rate of O_2 consumption of brain (Fig. 1, D) and kidney slices (C). The immediate effect on brain is to increase the rate slightly.
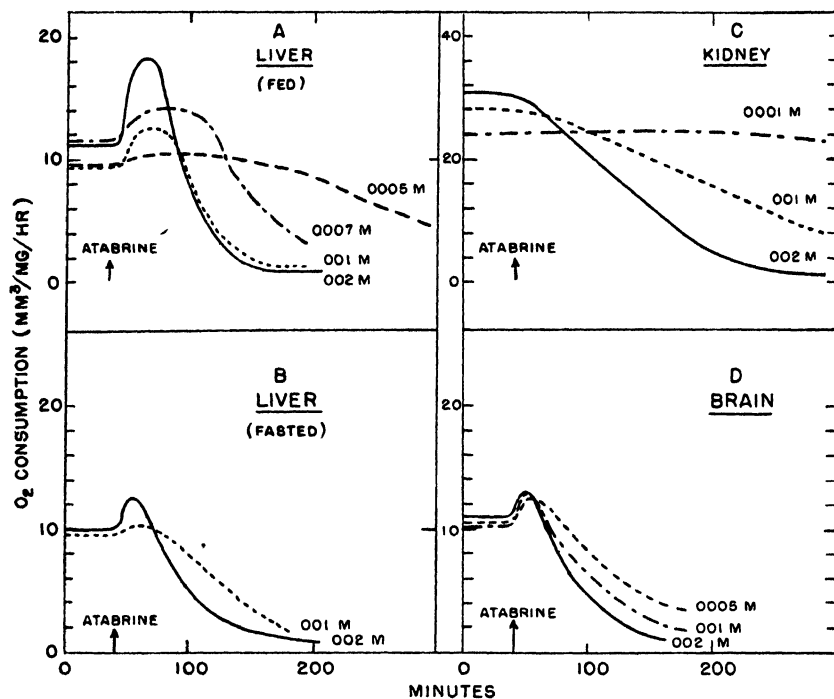
Fig 1. The inhibition of the O_2 consumption of rat tissue slices by atabrine.   The molar concentration of atabrine is given for each curve

Fig. 2 shows that liver and brain slices are able to catalyze the oxidation of p-phenylenediamine after being heavily treated with atabrine.

Neither lactate, pyruvate, citrate, fumarate, nor malate is able to restore the O_2 consumption of tissues after thorough treatment with atabrine. However, the addition of succinate after treatment with atabrine causes a sharp rise in O_2 consumption (Fig. 3).   With liver, the excess O_2 consumed is very nearly equivalent to the theoretical amount required to convert the succinate to fumarate.

Atabrine inhibits the oxidation of dl-alanine by Krebs' d-amino acid oxi-
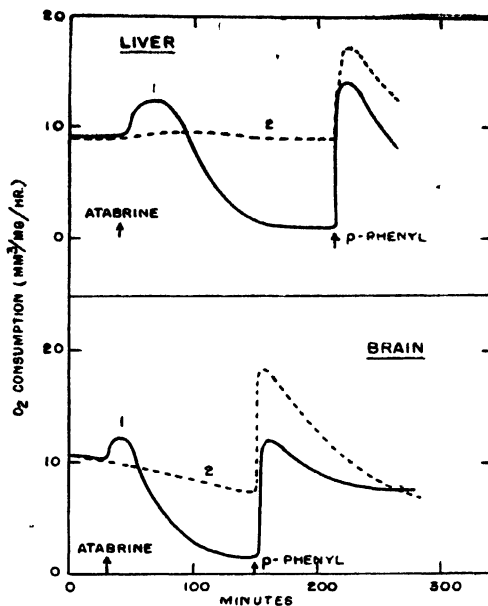
FIG 2. The oxidation of *p*-phenylenediamine (0.01 M) by tissue slices after treatment with atabrine (0.001 M). Curve 1 represents the O₂ consumption of tissues treated with atabrine and *p*-phenylenediamine, and Curve 2 those treated with *p*-phenylenediamine alone.
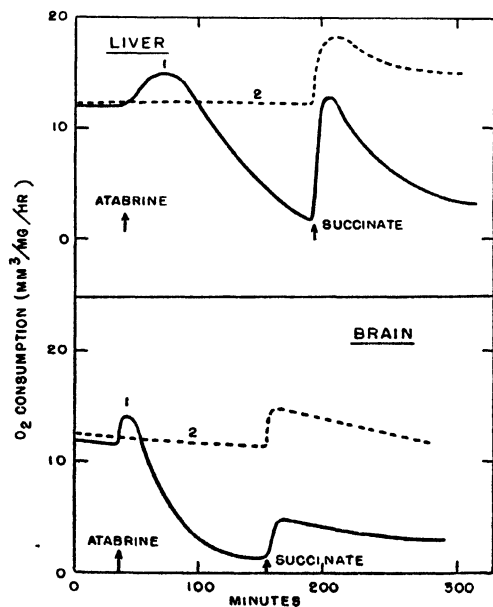
FIG. 3. The oxidation of succinate (0.02 M) by tissue slices after treatment with atabrine (0 001 M). Curves 1 and 2, as in Fig. 2.

dase (KE), as shown in Fig. 4.   The alanine was added from a side arm into
the flask containing 0.5 ml. of the enzyme preparation, atabrine at the de-
sired concentration, and sufficient 9 per cent pyrophosphate buffer (pH 8.2)
to make a final volume of 3.0 ml. 5×10^{-4} M atabrine definitely retards the
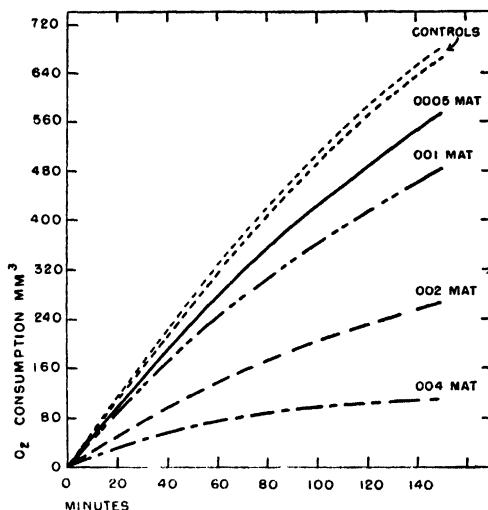
Fig. 4. The inhibition of d-amino acid oxidase by atabrine   The molar concen-
tration of atabrine (MAT) is indicated on each curve   The substrate was dl-alanine
(0.007 M)
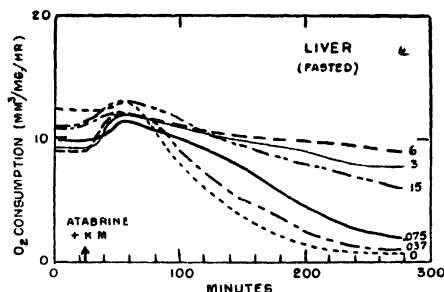
FIG. 5   The protection of liver slices against atabrine (0.001 M) by the prosthetic
group of d-amino acid oxidase   The amount of prosthetic group (KM) is given for each
curve as gm. of dried kidney from which it was prepared.

enzymic activity under these conditions.   The inhibition can be partially
offset by adding the prosthetic group prepared by heat (KH) or methanol
(KM) treatment of the enzyme preparation.

The addition of the prosthetic group of the d-amino acid oxidase prepara-
tion, as derived by heat or methanol treatment, protects tissue slices against

the toxic effects of atabrine (Fig. 5). In the experiment shown, 0.001 M atabrine and KM were tipped onto the liver slices at 25 minutes. The amount of KM added is indicated for each curve as gm. of dried kidney from which it was prepared. Complete protection against atabrine is afforded by the prosthetic group from 0.3 to 0.6 gm. of kidney under the conditions of the experiment.

<div style="text-align:center">DISCUSSION</div>

Martin and coworkers state (2) that atabrine, at a concentration of 3 mg. per 100 mg. of tissue, has no effect on the O_2 consumption of brain or other organs. This would represent a concentration of approximately 2×10^{-3} M, assuming a volume of 3.0 ml. in their experiments. We were able to demonstrate almost complete inhibition of brain respiration at less than 5×10^{-4} M concentration of atabrine.

The fact that tissues are able to catalyze rapidly the oxidation of p-phenylenediamine or succinate, after thorough treatment with atabrine, shows that the inhibition of O_2 consumption is probably not due to interference with the cytochrome or the cytochrome oxidase of the respiratory mechanism. The oxidation of succinate also shows that succinic dehydrogenase is not blocked by the concentrations of atabrine used.

The other known links in the respiratory chain are the pyridino-protein enzymes and the yellow enzyme, cytochrome reductase (8). There is no evidence as to the effect of atabrine on the pyridino-protein enzymes but the fact that it inhibits d-amino acid oxidase indicates that the interference with respiration might be through the yellow enzymes. The proof of this possibility would require the examination of the effects of atabrine on a respiratory system comprised of isolated enzymes. Since such a program was precluded by commitments on other problems, the results, as presented here, were communicated to Dr. Haas. The results of his investigation are given in the following paper.

<div style="text-align:center">SUMMARY</div>

Atabrine inhibits the O_2 consumption of rat liver, brain, and kidney slices. After such inhibition the tissues are unable to oxidize glucose, lactate, pyruvate, malate, citrate, or fumarate, but can oxidize succinate with a consumption of O_2 equivalent to a conversion to fumarate. After treatment with atabrine the tissues catalyze the oxidation of p-phenylenediamine. Atabrine also inhibits d-amino acid oxidase. The prosthetic group of this enzyme prevents the fall in O_2 consumption due to atabrine. The indications are that atabrine interferes with the yellow enzyme systems.

BIBLIOGRAPHY

1. Fulton, J. D., and Christophers, S. R., *Ann. Trop. Med. and Parasitol.*, **32**, 77 (1938).
2. Martin, S. J., Cominole, B., and Clark, B. B., *J. Pharmacol. and Exp. Therap.*, **65**, 156 (1939).
3. Molitor, H., Publication of the American Association for the Advancement of Science, **15**, 261 (1941).
4. Wright, C I , and Lillie, R. D., *Pub. Health Rep.*, *U. S. P. H. S* , **58**, 1242 (1943).
5. Dickens, F., and Greville, G. D., *Biochem. J.*, **29**, 1468 (1935)
6. Krebs, H. A , *Biochem. J.*, **29**, 1620 (1935).
7. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **81**, 389 (1929)
8. Haas, E , Horecker, B. L , and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940).

THE EFFECT OF ATABRINE AND QUININE ON ISOLATED RESPIRATORY ENZYMES

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Oxygen consumption and utilization of glucose appear to play an important rôle in cultivation and survival of malaria parasites (1–4). Antimalarial drugs effectively inhibit respiration of the parasite (5, 6) and comparable inhibition of the respiration of mammalian tissue by atabrine (7) has been reported by Wright and Sabine.[1] It is conceivable that the inhibition of parasite respiration observed *in vitro* is indicative of a similar process taking place in the body when the drug is therapeutically effective. Therefore it seemed desirable to investigate the action of antimalarials on the isolated components of the respiratory enzyme system. Knowledge of the effects of these important drugs on enzymes of known structure may offer a better understanding of the mechanisms of therapeutic activity and toxicity.

In Fig. 1 the components of the respiratory system are arranged in the order in which they react with each other, with molecular oxygen, and with the substrate. By selecting proper concentrations conditions can be so adjusted that any reaction in this scheme becomes the rate-determining factor. Specific tests were developed, by use of spectrophotometric and manometric methods, permitting precise investigation of the action of the drug on each component. Detailed descriptions of the tests are given to facilitate studies on the effect of therapeutic agents on various components of the respiratory system. Additional information may be gained by considering intermediate steps rather than by studying over-all reactions.

Inhibition of Cytochrome Oxidase—The enzyme was prepared according to the new procedure (8). Enzymatic activity and inhibition were measured in Warburg manometers after incubation at 25° in 0.05 M phosphate buffer of pH 7.1. Incubation of the enzyme with atabrine for 15 minutes or for 8 hours produced the same inhibition. The effect of atabrine and of quinine on cytochrome oxidase is shown in Table I.

Under these conditions the oxygen consumption is proportional to the

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[1] Personal communication from Dr. C. I. Wright, who also kindly supplied a sample of atabrine.

MOLECULAR OXYGEN
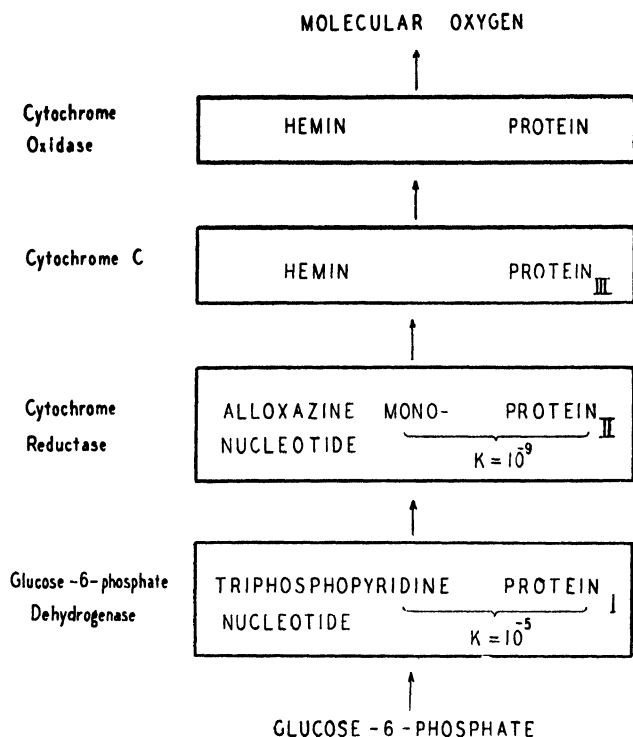
FIG. 1. Respiratory system

TABLE 1

Determination of Cytochrome Oxidase Activity, Inhibition by Atabrine and Quinine

2.5 cc. of 0.05 M phosphate buffer, pH 7 1, + 1 0 mg of cytochrome c + 3.0 mg of hydroquinone; temperature, 25°; gas phase, air

	Experi- ment I	Experi- ment II	Experiment III	Experiment IV	Experiment V	Experiment VI
			Atabrine		Quinine	
			$0 2 \times 10^{-3}$ M	1×10^{-3} M	$0 5 \times 10^{-3}$ M	1×10^{-3} M
	cc	cc	cc	cc	cc	cc
Cytochrome oxidase .	0.05	0.10	0.10	0.10	0.10	0.10

Oxygen uptake in 15 min.

	c mm	c mm	c mm	c.mm	c mm	c mm.
	30	60	47	24	50	43
			per cent	per cent	per cent	per cent
Inhibition			22	60	17	28

enzyme concentration. Atabrine inhibits cytochrome oxidase 3 times as effectively as quinine. However, the inhibition is too small to account for the effect of atabrine on the respiration of malaria parasites. Oxidation of p-phenylenediamine occurs despite treating the tissue with atabrine, as shown in the preceding paper (7). This observation, however, does not rule out inhibition of cytochrome oxidase by atabrine. Oxidation of p-phenylenediamine can take place not only through the cytochrome oxidase system but also independently through a cyanide-insensitive catalyst, presumably cytochrome b (9).

Experiments with Cytochrome c—For determination of cytochrome activity a method similar to that of Keilin and Hartree (10) and Stotz, Harrer, Schultze, and King (11) was used. In this test cytochrome c is reduced by ascorbic acid and subsequently reoxidized in the presence of molecular

<div align="center">

TABLE II

Test for Cytochrome c Activity

</div>

2.0 cc. of 0.06 M phosphate buffer, pH 7 1, + 2.0 mg. of ascorbic acid; temperature, 25°; gas phase, air

	Experiment I	Experiment II	Experiment III	Experiment IV
Atabrine concentration, M				0.001
Cytochrome c, mg.	1.0	2.0	2.0	2.0
" oxidase, cc.	0.50	0.50	0.25	0.50
Oxygen uptake in 30 min.				
	c mm.	c.mm.	c.mm.	c.mm.
	20.5	41	40	40

oxygen and cytochrome oxidase. The rate of oxygen consumption is proportional to the concentration of cytochrome c and is independent of the oxidase concentration. The latter condition is important because atabrine will react with the oxidase. Cytochrome was incubated with and without atabrine in 0.05 M phosphate buffer of pH 7.1 but no inhibition took place even after 8 hours at 25°. Experimental details are given in Table II.

Inhibition of Cytochrome Reductase by Atabrine—Observations of Wright and Sabine on the oxygen consumption of tissue slices and on a crude preparation of d-amino acid oxidase indicated that atabrine interferes with yellow enzyme systems. In order to correlate observations *in vivo* and *in vitro* and in order to obtain a better understanding of the atabrine action, a more detailed study of reactions of cytochrome reductase with anti-

malarials was undertaken. Cytochrome reductase consists of a dissociating complex made up of a specific protein and a prosthetic group, alloxazine mononucleotide (vitamin B_2 phosphate) (12). Our experiments indicate that atabrine reacts irreversibly with the *free* protein of the enzyme. The antimalarial agent competes with the prosthetic group for the protein, forming an inactive drug-protein complex which accounts for the blocking of the pathway of respiration. This conclusion is supported by experiments in which the temperature and the concentrations of atabrine and of vitamin B_2 phosphate have been varied. The inhibition by atabrine depends considerably on the temperature. As expected, greater inhibition occurs at elevated temperatures because the enzyme complex dissociates to a greater extent, thereby increasing the concentration of the free protein. Furthermore, the inhibition depends on the concentrations of both atabrine and vitamin B_2 phosphate, because the relative proportion of prosthetic group to drug determines whether the enzymatically active vitamin-protein complex or the inactive drug-protein complex predominates.

Enzymatic activity and inhibition are determined by measuring the rate of reduction of cytochrome c in the specific cytochrome reductase test previously described (12). The rate of reaction is proportional to the concentration of cytochrome reductase, but it is independent of the concentration of the other components. The test substances, prepared as previously described (12), had been stored for 3 years at $0°$. A solution of cytochrome reductase (1×10^{-6} M) was incubated for 15 minutes (0.02 M phosphate buffer, pH 8.3; $25°$) with various concentrations of atabrine (4×10^{-5} to 1×10^{-3} M). Incubation was carried out with an enzyme solution of higher concentration, as cytochrome reductase is unstable in dilute solutions. For determination of enzymatic activity the solution was diluted 100-fold with phosphate buffer containing atabrine of the indicated concentration. Experimental details of the test are given in Table III.

The results of Table III indicate that the inhibition of cytochrome reductase depends to a considerable extent on the atabrine concentration. The effect of temperature has been investigated under similar conditions (Fig. 2).

The inhibition of cytochrome reductase at $25°$ is 11 times higher than that at $0°$. The instability of the isolated enzyme does not permit experimentation at an elevated temperature, but extrapolation to $38°$ indicates that about 50 per cent inhibition might be expected by 3×10^{-5} M atabrine at body temperature.

Antagonism between Atabrine and Alloxazine Mononucleotide—The competition between atabrine and the prosthetic group for the enzyme protein may be demonstrated in two ways. Inhibition occurs upon addition of increasing amounts of the drug to a constant amount of prosthetic group

(Table III). On the other hand inhibition can be prevented by supplying increasing amounts of prosthetic group at a constant drug concentration (Table IV).

TABLE III

Test for Cytochrome Reductase Activity; Inhibition by Atabrine

Wave-length, 550 mμ; length of absorption cell, 0.32 cm.; temperature, 25°; gas phase, air.

1.0 cc. of 0.012 M phosphate buffer, pH 7.1, + 0.02 mg. of triphosphopyridine nucleotide + 0.90 mg. of hexose monophosphate + 0.12 mg. of *Zwischenferment* + 1.2 mg. of cytochrome c.

	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V
Atabrine concentration, M			0.4×10^{-4}	2×10^{-4}	10×10^{-4}
Cytochrome reductase, M	0.5×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}
Rate of reduction of cytochrome c					
$\dfrac{\Delta \log \text{CyFe}^{+++}}{\Delta t}$, min^{-1}..	0.047	0.094	0.081	0.048	0.017
Inhibition, %			14	49	82
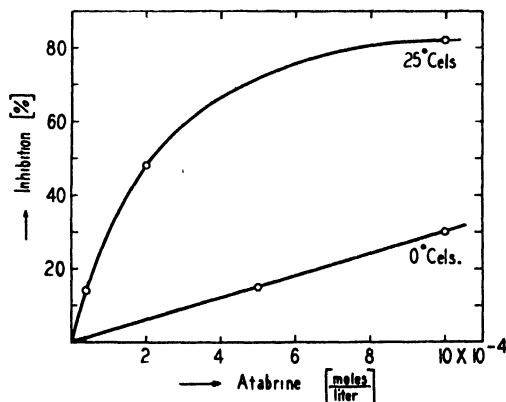
FIG. 2. Inhibition of cytochrome reductase as a function of temperature and atabrine concentration (enzyme incubated for 15 minutes).

Alloxazine mononucleotide was prepared by splitting cytochrome reductase with methanol, a method introduced by Theorell in studies on the old yellow enzyme (13). Methanol is added to a dialyzed solution of cyto-

chrome reductase and the denatured protein is removed by centrifugation. The supernatant solution is evaporated to dryness and the alloxazine mononucleotide redissolved in water. Cytochrome reductase was incubated in the presence of various amounts of prosthetic group (0 to 4×10^{-6} M) with 0.001 M atabrine (15 minutes, 25°, 0.02 M phosphate, pH 8.3). This enzyme solution was diluted 100 times with an atabrine-phosphate solution and the activity measured as described in the test for cytochrome reductase.

The results given in Table IV demonstrate that the inhibition by atabrine can be prevented by small amounts of alloxazine mononucleotide. The prosthetic group, when added in amounts comparable to those present in the enzyme, protects the protein; 1 molecule of prosthetic group is capable of neutralizing the action of about 500 molecules of the drug. This fact

TABLE IV

Competition between Atabrine and Alloxazine Mononucleotide

	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V	Experiment VI
Atabrine concentration, M		1×10^{-3}	1×10^{-3}	1×10^{-3}	1×10^{-3}	1×10^{-3}
Cytochrome reductase, M	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}	
Alloxazine mononucleotide, M			1×10^{-8}	2×10^{-8}	4×10^{-8}	4×10^{-8}
Rate of reduction of cytochrome c						
$\dfrac{\Delta \log \text{CyFe}^{+++}}{\Delta t}$, $min.^{-1}$	0.094	0.019	0.047	0.072	0.087	0.006
Inhibition, %		80	50	23	7	

indicates that the enzyme protein has a much greater affinity for the alloxazine mononucleotide than for atabrine. Consequently, it may be anticipated that some of the alloxazine derivatives may be effective antimalarials, acting by combining irreversibly with specific enzyme proteins. A vitamin B_2 analogue may take up the space but not the function of the vitamin, thus preventing respiration and growth of the parasite. The reaction of atabrine with cytochrome reductase is an irreversible process. Addition of the prosthetic group does not restore enzymatic activity after inhibition has taken place. Since the *in vitro* action of atabrine on respiratory enzymes can be counteracted by vitamin B_2 phosphate, the efficiency of the drug *in vivo* might be considerably enhanced by avoiding an excess of the vitamin in the diet during treatment of malaria.

Inhibition of Cytochrome Reductase by Quinine—Quinine was investigated

under the conditions described for atabrine and was found to be 12 times less effective than atabrine in inhibiting cytochrome reductase; e.g., 0.0005 and 0.001 M quinine inhibited only 14 and 30 per cent respectively.

Reaction with Glucose-6-phosphate Dehydrogenase—The enzyme which oxidizes glucose-6-phosphoric acid to phosphohexonic acid consists of a specific protein (*Zwischenferment* or Protein I in Fig. 1) and a prosthetic group, triphosphopyridine nucleotide (14, 15). The effect of antimalarials on the prosthetic group and on the protein has been investigated separately. The activity of triphosphopyridine nucleotide after incubation with atabrine in buffered solution was measured in the analytical test previously described (16).

The reaction rate (Table V) in the test is proportional to triphosphopyridine nucleotide and independent of the concentration of cytochrome reductase. Triphosphopyridine nucleotide is nòt inhibited by atabrine.

TABLE V

Determination of Triphosphopyrìdine Nucleotide Activity; Incubated with Atabrine

	Experiment I	Experiment II	Experiment III	Experiment IV
Atabrine concentration, M				0.001
Triphosphopyridine nucleotide concentration, M	$1\ 3 \times 10^{-7}$	2.6×10^{-7}	2.6×10^{-7}	2.6×10^{-7}
Cytochrome reductase, M	1×10^{-6}	0.5×10^{-6}	1×10^{-6}	1×10^{-6}
Rate of reduction of cytochrome *c*				
$\dfrac{\Delta \log \text{CyFe}^{+++}}{\Delta t}$, $min.^{-1}$	0.027	0.055	0.055	0.055

A new colorimetric test was introduced for studies on the protein moiety of the dehydrogenase because the strong absorption of ultraviolet light by atabrine interferes with ultraviolet spectroscopy. Experimental details of the test are given in the following paper (17). Atabrine strongly inhibits the action of this protein while quinine (0.001 M) has no effect. Addition of glucose-6-phosphate or triphosphopyridine nucleotide protects the protein to a certain extent against the inhibitory action of atabrine. The protein was incubated with 0.0005 M atabrine and 0.02 M phosphate, pH 8.3, for 15 minuteš at 25°, with addition of varying amounts of glucose-6-phosphate (0.0002 to 0.002 M). For determination of enzymatic activity the solution was diluted 10 times with phosphate buffer containing atabrine. Table VI demonstrates the inhibition by atabrine and the effect of glucose-6-phosphate.

Relatively high concentrations of the substrate, glucose-6-phosphate,

are required to protect the *Zwischenferment* from the inhibiting action of atabrine, while the prosthetic group, alloxazine mononucleotide, is 1000 times more effective in protecting its protein. This higher efficiency presumably is due to the greater stability of the flavin-protein complex ($K = 10^{-9}$ M) as compared with the glucose-6-phosphate-protein complex which is dissociated to a much greater extent ($K = 10^{-3}$ M). Competition between prosthetic group and atabrine for the enzyme protein has been demonstrated (Table IV) and the mechanism of atabrine action was interpreted as displacement of the prosthetic group by the antimalarial agent.

TABLE VI

Inhibition of Zwischenferment by Atabrine; Effect of Glucose-6-phosphate

Wave-length, 600 mμ; length of absorption cell, 1.0 cm.; temperature, 25°. 3.0 cc. of 0.015 M phosphate buffer, pH 8.3, 0.02 mg. of triphosphopyridine nucleotide, 0.02 mg. of 2,6-dichlorophenol indophenol, 3.0 mg. of *Zwischenferment*.

	Experiment I	Experiment Ia	Experiment II	Experiment IIa	Experiment III	Experiment IIIa
Atabrine concentration, M		5×10^{-4}		5×10^{-4}		5×10^{-4}
Glucose-6-phosphate concentration, M	0.2×10^{-4}	0.2×10^{-4}	1×10^{-4}	1×10^{-4}	2×10^{-4}	2×10^{-4}

Velocity of reaction (decolorization of 2,6-dichlorophenol indophenol); galvanometer deflection per 5 min.

	mm	*mm*	*mm*	*mm*	*mm*	*mm*
	66	15	112	64	111	85
		per cent		*per cent*		*per cent*
Inhibition		77		43		23

A second mechanism of atabrine action, namely displacement of the substrate by the drug, is suggested by the results of Table VI, which show that substrate and drug also compete for the enzyme protein. Antagonism between "essential metabolites" and drugs was suggested on the basis of experiments with living cells (18–20). This is demonstrated more clearly on isolated enzymes of known structure.

A summary of the effects of atabrine on respiratory enzymes is given in Table VII, together with results previously obtained on the respiration of malaria parasites (5) and on the respiration of mammalian tissues (7).

Among the known components of the respiratory system only cytochrome reductase and *Zwischenferment* need to be considered as possible points of interference by atabrine. This is brought out by the results in Table VII. The inhibitory effect of quinine on isolated enzymes is summarized in Table VIII and compared with that produced by equal concentrations of atabrine.

Quinine is much less effective than atabrine in inhibiting parasite respiration (5) and isolated respiratory enzymes. Therefore, the action of quinine as an antimalarial agent probably does not take place through interference with respiratory processes. Inhibition of the known respiratory catalysts would require a quinine concentration considerably in excess of the amounts normally used therapeutically.

TABLE VII

Effect of Atabrine on Isolated Respiratory Enzymes, on Parasite Respiration, and on Tissue Respiration

Enzymes	Temperature	Atabrine concentration	Inhibition
	°C	M	per cent
Cytochrome oxidase	25	10×10^{-4}	60
" c	25	10×10^{-4}	0
" reductase	25	2×10^{-4}	49
" "	(38)	$(0.3) \times 10^{-4}$	(50)
Triphosphopyridine nucleotide	25	10×10^{-4}	0
Zwischenferment (Protein I) ..	25	5×10^{-4}	77
Respiration of malaria parasites	38	1×10^{-4}	61
" " mammalian tissue	38	5×10^{-4}	61

The values in parentheses were obtained by extrapolation, while all other values in the table are experimental figures.

Very little is known about the respiratory mechanism of malaria parasites, although the following indirect evidence seems to indicate that it conforms with the general scheme of biological oxidations: Malaria *Plasmodia* utilize glucose and their respiration is inhibited by KCN (21), suggesting participation of a heavy metal-containing enzyme like Warburg's *Atmungsferment*. Finally, the respiration of parasites as well as of mammalian tissues can be inhibited by atabrine.

An attempt was made to obtain some of the known respiratory catalysts from isolated parasites in order to elucidate their respiratory mechanism. We are greatly obliged to Dr. William B. Wendel, University of Tennessee, for a generous gift of *Plasmodium knowlesi* which he had isolated from erythrocytes of heavily infected *Macaca mulatta* monkeys and dried *in vacuo*

at room temperature after extensive washing. The parasites were very stable and could not be disrupted by autolysis for a week at room temperature, by bacteriolytic action of lysozyme, by ultrasonic waves, by grinding with powdered glass, or even by heating to 90°. All these procedures as well as variation of pH, salt concentration, and time of autolysis failed to release cytochrome reductase or triphosphopyridine nucleotide, although very small amounts could have been detected with the analytical methods described. No disintegration of the parasites took place, as evidenced by microscopic investigation and by the observation that practically no protein was released during all these manipulations. Disintegration might occur in similar experiments with fresh parasites, which unfortunately were not at our disposal.

TABLE VIII

Comparison of Inhibition by Atabrine and by Quinine; Drug Concentration, 5 × 10⁻⁴ M

| Enzyme | Temperature | Inhibition by | | Relative effectiveness*
$\frac{Atabrine}{Quinine}$ |
		Atabrine	Quinine	
	°C.	*per cent*	*per cent*	
Cytochrome oxidase	25	40	17	3
" reductase	25	73	14	12
Zwischenferment (Protein I)	25	78	0	∞
Respiration of *Plasmodium knowlesi*	38	80	26	20

* At low concentrations of the drug at which inhibition is proportional to the drug concentration.

SUMMARY

1. A description of enzymatic tests is given which will facilitate the study of the effects of therapeutic agents on the components of the respiratory enzyme system.

2. The activity of cytochrome reductase and glucose-6-phosphate dehydrogenase is inhibited by low concentrations of atabrine. Cytochrome oxidase is less affected, while triphosphopyridine nucleotide and cytochrome *c* are not inhibited at all.

3. Inhibition of cytochrome reductase is caused by an irreversible reaction of atabrine with the free protein, whereby the drug competes with the prosthetic group of the enzyme. Antagonism between prosthetic group and antimalarial agent has been demonstrated in preventing drug action by addition of alloxazine mononucleotide.

4. The enzyme protein has a much greater affinity for the prosthetic

group than for atabrine, since 1 γ of vitamin B$_2$ phosphate will neutralize the action of about 500 γ of atabrine.

5. Atabrine inhibits glucose-6-phosphate dehydrogenase by reacting with the protein moiety of this enzyme. Addition of glucose-6-phosphate partially prevents and even reverses the inhibition by atabrine, which is evidence for a competition between substrate and drug.

6. Quinine is much less effective than atabrine as an inhibitor of respiratory catalysts. This suggests that the antimalarial action of quinine probably is not due to interference with the respiratory processes of the parasite.

We are particularly indebted to the Rockefeller Foundation for the financial support which has made this work possible and to Elisabeth Haas for her technical assistance.

BIBLIOGRAPHY

1 Bass, C. C., and Johns, F. M , *J. Exp. Med.*, **16,** 567 (1912).
2 Fulton, J D , *Ann Trop Med. and Parasitol.*, **33,** 217 (1939)
3. Coulston, F , *J Parasitol* , **27,** suppl., 38 (1941).
4. Trager, W., *J. Exp. Med* , **77,** 411 (1943).
5 Fulton, J D , and Christophers, S R., *Ann. Trop. Med. and Parasitol.*, **32,** 77 (1938)
6 Coggeshall, L T., and Maier, J , *J Infect. Dis* , **69,** 108 (1941).
7 Wright, C I , and Sabine, J C , *J Biol Chem* , **155, 315** (1944).
8 Haas, E., *J Biol Chem* , **148,** 481 (1943).
9 Stotz, E , Sidwell, A. E , Jr , and Hogness, T. R., *J. Biol Chem.*, **124, 733** (1938).
10 Keilin, D., and Hartree, E F , *Proc Roy. Soc. London, Series B*, **125,** 171 (1938).
11 Stotz, E , Harrer, C. J., Schultze, M O , and King, C. G , *J. Biol. Chem.*, **122,** 407 (1937–38).
12 Haas, E , Horecker, B L , and Hogness, T. R., *J. Biol Chem* , **136,** 747 (1940).
13. Theorell, H., *Biochem Z.*, **278,** 263 (1935).
14 Warburg, O., Christian, W., and Griese, A., *Biochem. Z* , **282,** 157 (1935).
15. Negelein, E , and Haas, E , *Biochem. Z.*, **282,** 206 (1935).
16. Haas, E., Harrer, C. J., and Hogness, T. R., *J Biol Chem.*, **142,** 835 (1942).
17. Haas, E , *J. Biol Chem* , **155,** 333 (1944).
18 Woods, D. D , *Brit. J Exp. Path.*, **21,** 74 (1940).
19 Dorfman, A., Rice, L , Koser, S. A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, **45,** 750 (1940).
20 McIlwain, H , *Lancet*, **1,** 412 (1942).
21. Christophers, S R., and Fulton, J. D., *Ann. Trop. Med. and Parasitol.*, **32, 43** (1938).

A COLORIMETRIC DETERMINATION FOR STUDIES INVOLVING COENZYMES

MICRODETERMINATION OF GLUCOSE-6-PHOSPHATE

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Ultraviolet spectroscopy, which has been used so extensively in studies on the pyridine nucleotides (1–3), cannot be employed under all conditions. For example, certain antimalarial drugs, reported in the preceding paper, are colored substances which strongly absorb ultraviolet light. This difficulty was overcome by introducing a color reaction based upon the reduction of 2,6-dichlorophenol indophenol by dihydrocoenzymes. This new method makes feasible the investigation of coenzymes, dehydrogenases, and substrates in the visible region of the spectrum and offers certain advantages over the ultraviolet test. (1) Smaller amounts of the pyridine nucleotides are sufficient in this assay because of the catalytic function of the coenzyme, whereas stoichiometric amounts were previously required. (2) The sensitivity of the optical determination is increased because the light absorption of the dye is higher than that of the coenzyme. (3) A simple colorimeter with a red light filter can now be used instead of a spectrophotometer required for the ultraviolet region, since the dye has a broad absorption band between 560 and 640 mμ.

In this paper conditions for the quantitative determination of glucose-6-phosphate and *Zwischenferment* are described. The dye is readily reduced and decolorized in the following system: glucose-6-phosphate-*Zwischenferment*-triphosphopyridine nucleotide-2,6-dichlorophenol indophenol. This colorimetric method should also be useful in the study of reactions involving diphosphopyridine nucleotide.

Determination of Zwischenferment

In the experiments reported in Table I conditions are so arranged that the rate of decolorization of the dye is proportional to the concentration of *Zwischenferment*. All components except *Zwischenferment* were added to the cell and a few minutes allowed for temperature equilibrium. Then the *Zwischenferment* was added and decolorization of the dye measured photometrically. The galvanometer deflection after addition of *Zwischenferment*

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is directly proportional to the enzyme concentration for any time interval up to 7 minutes. No further calculation is required for the determination of enzyme concentration because of this direct proportionality. Autoxidation of the dye is negligible; hence no precautions against the presence of air

TABLE I

Analytical Method for Zwischenferment

Wave-length, 600 mμ; temperature, 25°; length of absorption cell, 1 0 cm. 3.0 cc. of 0.015 M phosphate buffer, pH 8 3, 1.0 mg. of glucose-6-phosphate, 0.02 mg of triphosphopyridine nucleotide, 0 02 mg. of 2,6-dichlorophenol indophenol, 3 0 mg. of *Zwischenferment*.

Time	Galvanometer reading	Velocity (galvanometer deflection per 5 min)
min.	*mm.*	*mm.*
0	172	
5	312	$312 - 172 = 140$
10	400	
15	415	
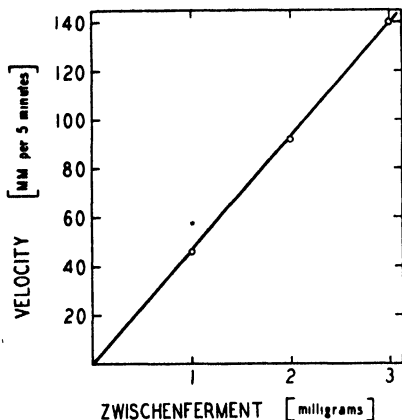
FIG. 1,   FIG. 2

FIG. 1. Decolorization of 2,6-dichlorophenol indophenol as a function of *Zwischenferment*. Velocity as the galvanometer deflection in mm. per 5 minutes.
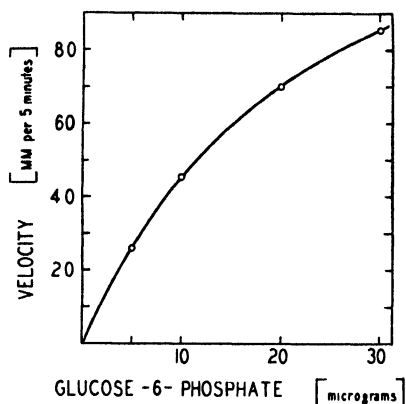
FIG. 2. Decolorization of 2,6-dichlorophenol indophenol as a function of glucose-6-phosphate. Velocity as the galvanometer deflection in mm. per 5 minutes.

need to be taken. Glucose-6-phosphate, triphosphopyridine nucleotide, and *Zwischenferment* were prepared as previously described (4); 2,6-dichlorophenol indophenol was a commercial product supplied by the La-Motte Chemical Products Company. The velocity of decolorization

(galvanometer deflection for the first 5 minutes) as function of the *Zwischenferment* concentration is shown in Fig. 1.

Analytical Method for Glucose-6-phosphate

The method as described in Table 1 may be used also for the microdetermination of glucose-6-phosphate. The velocity of reduction of 2,6-dichlorophenol indophenol as a function of glucose-6-phosphate is plotted in Fig. 2.

SUMMARY

1. A spectrophotometric method is described which permits the investigation of reactions involving pyridine nucleotides (coenzymes I and II).

2. Advantages of the new method over the ultraviolet test are pointed out.

3. Applications of the method are presented including a microdetermination of glucose-6-phosphate which requires only about 10 γ of substance.

BIBLIOGRAPHY

1. Warburg, O., Christian, W, and Griese, A , *Biochem Z.*, **282,** 157 (1935)
2. Negelein, E., and Haas, E., *Biochem Z*, **282,** 206 (1935).
3. Negelein, E, and Wulff, H. J , *Biochem. Z.*, **293,** 351 (1937).
4 Haas, E., Horecker, B. L., and Hogness, T. R , *J Biol. Chem*, **136,** 747 (1940)

A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS

III. CHANGES IN THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE LIVER AND KIDNEYS DURING DEFICIENCY*

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Previous investigations (1, 2) had revealed that guinea pigs raised on a diet lacking in the antistiffness factor (3) developed a typical deficiency disease. The first outward sign of the deficiency was the development of a stiffness at the wrist joint. As the disease progressed this symptom increased in severity. Upon autopsy of animals which had been on the deficient diet for a year or longer, large deposits of Ca, mainly in the form of calcium triphosphate, were found in connection with almost any body tissue, conspicuous locations being muscle, subcutaneous tissue, aorta, liver, and kidneys. This abnormal deposition of calcium phosphate suggested a derangement of the phosphorus metabolism. For this reason an investigation was made of the distribution of the acid-soluble phosphorus in the liver and kidneys of normal and deficient guinea pigs. The following fractions were determined: (1) total P; (2) inorganic P; (3) easily hydrolyzable P (15 minutes hydrolysis with N sulfuric acid in a boiling water bath) accounting for 67 per cent of the adenosine triphosphate plus 50 per cent of the adenosine diphosphate; (4) alcohol-insoluble P, compounds yielding Ba salts insoluble in 80 per cent alcohol (mostly glycerophosphate and glucose phosphate), (5) mercuric-insoluble P, containing all the stable nucleotide P, (6) alcohol-soluble P, representing compounds yielding Hg and Ba salts soluble in 80 per cent alcohol.

EXPERIMENTAL

Guinea pigs 11 to 12 weeks of age and weighing from 300 to 350 gm. were segregated as to sex and housed in clean cages on autoclaved straw The animals were fed *ad libitum* a diet of the following composition: skim milk powder 16 gm., copper sulfate 0.25 mg., ferric chloride 0.25 mg., and water 84 gm. The average daily food intake was 280 gm ,

approximately evenly divided over the morning and night feeding. Food was prepared twice a day. To the morning diet was added a solution of the water-soluble vitamins in such a concentration that the average daily vitamin intake was as follows: thiamine hydrochloride 0.2 mg., riboflavin 0.5 mg., pyridoxine hydrochloride 0.1 mg., nicotinic acid 1 mg., Ca pantothenate 0.1 mg., inositol 10 mg., p-aminobenzoic acid 2 mg., choline 50 mg., and biotin (S. M. A. Concentrate S-200) 0.01 mg. A solution of the fat-soluble vitamins in cottonseed oil was added to the evening food. The average daily intake of these vitamins was β-carotene 150 I.U., viosterol 40 I.U., α-tocopherol 0.1 mg., 2-methyl-1,4-naphthoquinone 0.1 mg. Once a week 50 mg. of crystalline l-ascorbic acid, dissolved in water immediately before use, were administered by mouth. This amount is well above the minimum weekly dose recommended by Zilva (4). Water and iodized salt were provided *ad libitum*. The control animals received a stock diet composed of rolled barley, greens, water, and iodized salt *ad libitum*. The body weights of the guinea pigs were recorded twice a week throughout the course of the experiments. The animals on the deficient diet gained as regularly in weight as the stock animals and showed no signs of any deficiency diseases, except a constantly increasing stiffness at the wrist joint.

Animals were sacrificed at various intervals and the distribution of the acid-soluble phosphorus in the liver and kidneys was determined according to the method of Rapoport *et al.* (5) Wherever possible groups of fifteen guinea pigs were used for analysis.

The data were analyzed statistically according to the methods of Fisher (6).

Results

In Table I are represented the mean concentrations of the total acid-soluble phosphorus and of its fractions in the livers of guinea pigs receiving (a) the stock diet, (b) the deficient skim milk diet, and (c) the deficient skim milk diet supplemented with the antistiffness factor.[1] The most significant change took place in the easily hydrolyzable phosphorus. This fraction showed a decrease of close to 50 per cent after the animals had received the deficient diet for 1 week. The lower limit of this fraction appeared to be 3 4 mg. per 100 gm. of liver and was reached after a year. The total acid-soluble P fluctuated during the first weeks of deficiency. These fluctuations can be accounted for by the changes occurring in the other fractions. During the later stages of the deficiency the total acid-

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[1] This factor was administered as a solution in cottonseed oil of the crystalline material (100,000,000 units per gm) The preparation of this material will be described in a later publication.

soluble P showed a significant increase which was caused by the increase in the inorganic P, mercuric-insoluble P, and alcohol-soluble P. The alcohol-insoluble P fluctuated slightly and seemed to be lower than normal in the later stage of the deficiency.

The last two horizontal rows of Table I represent the values of the different phosphorus fractions in the livers of guinea pigs receiving the

TABLE I

Distribution of Acid-Soluble Phosphorus in Liver of Normal and Deficient Guinea Pigs

Age	Diet	Time on diet	No of deter- mina- tions	Mean and s e , mg per 100 gm						
				Total acid- soluble P	Inor- ganic P	Easily hydro- lyzable P	Alcohol- insoluble P	Mercuric insoluble P	Alcohol- soluble P	Sum of fractions
wks		*wks*								
14	Stock	14	15	111 9	15 4	14.2	24 9	46 6	12 8	113.9
				±2.0	±0 7	±0 9	±0.5	±0 7	±0.7	±2 2
65	"	65	9	105 5	18.9	16.6	17 9	45 5	10.6	109.5
				±2.0	±0.8	±2 0	±0 8	±0 7	±1.0	±2.2
14	Deficient	1	15	103 8	27 4	7 9	20.5	40 4	9 7	106.5
				±0.8	±0 9	±0.1	±0 8	±0 5	±0 5	±0 6
15	"	2	15	122 0	26 6	5 4	25 9	44 4	16.4	118.8
				±2 7	±1 8	±0 7	±0.9	±1 5	±0 7	±2.8
16	"	3	11	128 9	25 8	6 1	23 9	52 1	20 9	123.4
				±2 8	±0.7	±0 6	±0 9	±2 4	±0.4	±0 4
20	"	7	8	118 9	24 6	4.9	19.3	55 1	15.4	119.4
				±4 0	±1 2	±0.6	±1.9	±2 0	±0.9	±3.7
11	"	28	11	131.6	30 7	4.0	20 5	56 4	18 0	129 6
				±1.9	±1 5	±0.2	±0.5	±0.7	±0.5	±1.9
70	"	57	16	165.6	36 6	3 4	19.3	79 5	26.3	165.1
				±1.5	±0.9	±0 3	±0 4	±0.6	±0 4	±1.4
17	" + a s f *	1	14	110.5	21.7	12 7	22 3	42 8	10 4	109.9
				±1 2	±0 9	±0 9	±0 6	±1 2	±0.6	±1.3
23	" "	10	14	112 6	24 8	14 2	19 1	43 3	10 9	111.3
				±2 4	±1 0	±0 9	±1.1	±0.6	±1 1	±2.7

* 1000 units of the antistiffness factor every other day during the whole course of the experiment

skim milk diet supplemented with the antistiffness factor (1000 units every other day). It will be noted that the distribution is essentially normal.

The values for the distribution of acid-soluble phosphorus in the kidneys of the same animals are given in Table II. Here the same trend as in the livers is apparent. The inorganic P, however, showed a greater increase during the deficiency. In contrast with the increase in the mercuric-insoluble P found in the liver, this fraction remained constant during the deficiency disease.

Since it was shown that an abnormal distribution of the acid-soluble phosphorus could be prevented by the continued administration of the antistiffness factor, it was of interest to determine whether this factor could restore the abnormal distribution to normal. A group of fifteen guinea pigs was kept on the deficient diet for 7 weeks. During the last 5 days of the experiment seven animals of this group received 1000 units

TABLE II

Distribution of Acid-Soluble Phosphorus in Kidneys of Normal and Deficient Guinea Pigs

Age	Diet	Time on diet	No of determinations	Mean and S E , mg per 100 gm						
				Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric insoluble P	Alcohol-soluble P	Sum of fractions
wks		wks								
14	Stock	14	15	95 2	15 1	14 0	15.6	45 2	12 6	102 5
				±0 2	±0 1	±0 3	±0.1	±0.6	±0.9	±1.7
65	"	65	9	100 4	26 1	10 6	17 9	27 9	11 2	93.7
				±2 0	±1 2	±0 5	±0 9	±1 0	±0 2	±0 9
14	Deficient	1	15	95.8	26 6	7 8	8.8	41.8	15.1	100 1
				±1 0	±0 1	±0 1	±0 1	±0.1	±0 1	±0.9
15	"	2	15	107 8	27 9	3 1	15 9	41.4	16 5	104.8
				±0 9	±0 1	±0 1	±0 1	±1.1	±0.1	±1.9
16	"	3	11	117 3	30 2	3 6	24 4	40 2	16 3	114.7
				±0 1	±0 1	±0 1	±0 1	±0 1	±0 1	±1 3
20	"	7	8	112 6	32 6	3 3	20 1	40 6	16 1	112.7
				±2.9	±1 0	±0 1	±0 7	±0 3	±0 9	±1.9
41	"	.28	11	129 3	50 0	3 6	16 8	44.7	11 3	126.4
				±3 5	±2.5	±0 3	±0 2	±1.0	±0.2	±3 0
70	"	57	16	153 2	65.3	2 3	17.6	43.4	19 2	147 8
				±2 2	±2 0	±0 2	±0 2	±0.9	±0 4	±2.9
17	" + a s f *	4	14	112.0	27.1	11 9	16.6	43.4	14 2	113.2
				±0 7	±0 6	±0.4	±0 6	±0 9	±0 4	±0 4
23	" "	10	14	103 9	26.2	10.8	15.7	39 9	11.1	104.2
				±0 8	±0 7	±0 1	±0.1	±0 1	±0.1	±1.5

* 1000 units of the antistiffness factor every other day during the whole course of the experiment.

of the antistiffness factor per day. At the end of the 5 days all the animals were sacrificed and the distribution of acid-soluble phosphorus in the liver and kidneys determined. The results of this experiment are represented in Table III.

From these it is clear that administration of the antistiffness factor to animals with a low easily hydrolyzable P results in a steep increase in this level, which is comparable to the equally sharp decrease during the onset

of the deficiency. This fraction returned to normal after the administration of 1000 units per day for the last 5 days of the experiment. The mercuric-insoluble P also returned to the normal level.

TABLE III

Effect of "Cure" with Antistiffness Factor on Distribution of Acid-Soluble Phosphorus in Liver and Kidneys of 20 Week-Old Deficient Guinea Pigs on Diet for 7 Weeks

	Diet	No of determinations	Mean and s e., mg. per 100 gm.						
			Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
Liver	Deficient	8	118.9	24.6	4.9	19.3	55.1	15.4	119.4
			±4.0	±1.2	±0.6	±1.9	±2.0	±0.9	±3.7
	" + a.s.f *	7	110 6	22.6	13.2	15.9	44.7	15.8	112.2
			±3.4	±1.3	±0.8	±0.7	±0.9	±0.9	±1.9
Kidneys	Deficient	8	112 6	32.6	3.3	20.1	40.6	16 1	112 7
			±2.9	±1.0	±0.1	±0 7	±0.3	±0.9	±1 9
	" + a s.f *	7	108 1	24.0	9.3	18.5	39.7	16.3	107.8
			±2.9	±0.1	±0.5	±1.8	±0.1	±0.9	±0.6

* 1000 units of the antistiffness factor for the last 5 days.

TABLE IV

Comparison between Distribution of Acid-Soluble Phosphorus in Liver and Kidneys of 65 Week-Old Guinea Pigs on Stock Diet and Raw Milk Diet

	Diet	Time on diet	No of determinations	Mean and s e., mg per 100 gm.						
				Total acid-soluble P	Inorganic P	Easily hydrolyzable P	Alcohol-insoluble P	Mercuric-insoluble P	Alcohol-soluble P	Sum of fractions
		wks								
Liver	Stock	65	9	105.5	18.9	16.6	17.9	45.5	10.6	109.5
				±2 0	±0.8	±2.0	±0.8	±0.7	±1.0	±2.2
	Raw milk	52	6	112.4	22.6	11.3	18.3	43.1	14.9	110.2
				±1.8	±1.2	±0.6	±0.7	±0.8	±0.7	±1 1
Kidneys	Stock	65	9	100.4	26.1	10.6	17.9	27.9	11.2	93.7
				±2.0	±1.2	±0 5	±0 9	±1.0	±0.2	±0.9
	Raw milk	52	6	154.2	79.3	2.4	19.1	49.1	11.5	161.4
				±1.1	±1.2	±0.5	±0.9	±0.6	±0.5	±1.1

It was noted previously by Wulzen that guinea pigs very rarely developed wrist stiffness when raised on raw milk. It was therefore of considerable interest to compare the distribution of the acid-soluble phosphorus in the livers and kidneys of raw milk-fed animals with that in the same organs of the old "stock" animals. A small number of the animals on raw milk were available for the determination. The results are shown in Table IV.

The distribution of the acid-soluble P in the liver of these animals is comparable to that in the livers of the stock animals. A somewhat higher inorganic P content may be accounted for by the high P content of the skim milk diet. There is, however, a large difference in the distribution of the acid-soluble P in the kidneys. Here the total P and the inorganic P were very high, while the easily hydrolyzable P has decreased to the low level of 2.4 mg. per 100 gm.

<div align="center">DISCUSSION</div>

The interpretation of the changes in the distribution of the acid-soluble phosphorus in the liver and kidneys during the course of the deficiency is as yet difficult. The results indicate a derangement in the phosphorus metabolism. The changes in the total P during the onset of the deficiency can be explained in the main by the opposing trends of the inorganic P on the one hand and of the last four fractions on the other. The inorganic P showed a constant increase during the deficiency. The most significant changes, however, occurred in the easily hydrolyzable P and the mercuric-insoluble P. These two fractions seem to be closely connected. The easily hydrolyzable P fraction represents about 67 per cent of the adenosine triphosphate plus 50 per cent of the adenosine diphosphate, while the mercuric-insoluble P fraction accounts for all the stable nucleotide P. It is the first fraction which responded almost immediately to the lack of the antistiffness factor in the diet and which was the first one to return to normal after this factor was administered. Rapoport (personal communication) has found that the changes in the easily hydrolyzable P during fasting indeed reflect a changing mixture of nucleotides, adenylic acid increasing at the expense of adenosine triphosphate. It is, therefore, possible that the changes in the adenosine triphosphate and the adenosine diphosphate are of prominence during the deficiency, perhaps involving the whole metabolism of the purines.

During this investigation it became apparent that the first noticeable outward symptom of the deficiency of the antistiffness factor, stiffness at the wrist joint, is not an essential feature in the early stages of the deficiency. Several animals did not show any wrist stiffness at all during the time limit of the experiment. The distribution of the acid-soluble phosphorus in these guinea pigs, however, was no different from that in "stiff" animals of the same experimental series.

<div align="center">SUMMARY</div>

The distribution of the acid-soluble phosphorus in the liver and kidneys of guinea pigs on a stock diet and on a diet mainly composed of skim milk powder, supplemented with the known vitamins, has been determined.

The concentration of the easily hydrolyzable P in both organs was found to be very markedly decreased during the deficiency. The inorganic P showed a sharp increase in the liver and kidneys. The increase of the mercuric-insoluble P in the liver seemed to be correlated with the decrease in the easily hydrolyzable P. The total acid-soluble P also was increased during the deficiency. The distribution of the acid-soluble P in the kidneys during the deficiency showed the same trends except that there was no appreciable increase in the mercuric-insoluble P. The antistiffness factor will prevent an abnormal distribution of the acid-soluble P or restore it to normal after it has developed. A possible interpretation of the cause of the abnormal distribution of the acid-soluble P has been presented.

BIBLIOGRAPHY

1 Wulzen, R , and Bahrs, A M , *Proc Am Physiol. Soc* , *Am J Physiol* **53,** P312 (1941)

2 van Wagtendonk, W J , and Wulzen, R , *Arch Biochem* , **1,** 373 (1943).

3 van Wagtendonk, W J , Schocken, V , and Wulzen, R , *Arch Biochem* , **3,** 305 (1944)

4 Zilva, S S , *Biochem J* , **35,** 1240 (1941)

5 Rapoport, S , Leva, E and Guest, G M , *J Biol Chem* , **149, 57** (1943)

6 Fisher, R A , Statistical methods for research workers Edinburgh 7th edition (1938)

A PRODUCT OF OXIDATIVE METABOLISM OF PYRIDOXINE, 2-METHYL-3-HYDROXY-4-CARBOXY-5-HYDROXY-METHYLPYRIDINE (4-PYRIDOXIC ACID)*

I. ISOLATION FROM URINE, STRUCTURE, AND SYNTHESIS

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In 1941 Singal and Sydenstricker (1) published briefly the following observation: If human urine, collected after the ingestion of pyridoxine, was filtered through a zeolite column, a substance was adsorbed which could be eluted from the column with neutral 25 per cent KCl solution and transferred to an isobutanol extract; when illumined by ultraviolet light, the latter exhibited a characteristic fluorescence. This observation was recently confirmed in these laboratories (2), and some evidence was presented that the substance in the urine responsible for this fluorescence is identical with an oxidation product of pyridoxine obtained by the action of permanganate on synthetic vitamin B_6. On the basis of certain analytic data, it was stated that the structure of this product is 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (I) which can be transformed by heating with acid into the lactone form (II).

In this communication there are presented the experimental details of the isolation of the urinary metabolite, the synthesis of the oxidation product of pyridoxine, and proof of its structure. Since the development of this work depended upon the use of a simple fluorometric technique for the identification and the quantitative estimation of the metabolite and of its derivative, a description of this technique is given first. In the description of the method the two fluorescent compounds are designated as the metabolite (I) and its lactone (II), although the proof of their structure and identification are given in a later portion of the paper.

EXPERIMENTAL

Fluorometric Determination of Metabolite (I) *and Lactone* (II)—The fluorescence measurements were made in the Coleman model 12 electronic

photofluorometer equipped with Filters B1 and PC1, employed for thio-chrome determinations; the galvanometer was set at 100 with a solution containing 0.3 γ of quinine sulfate per ml. of 0.1 N H_2SO_4. The aqueous solutions were diluted to bring them within the range of the instrument and the reaction adjusted to pH 3 to 4 for the metabolite (I) usually with glacial acetic acid, and for the lactone (II) to pH 9 $\pm$ 0.3 by the addition of solid sodium borate. Since the lactone form (II) gives a fluorescence intensity 25 times that of the metabolite (I) (see Table I and Fig. 1), the latter was determined preferably as the lactone, after being heated in 1.0 N HCl for 15 minutes at 100°. For urine, a convenient procedure is to heat 1.00 ml. of urine with 9 ml. of 1:10 solution of concentrated HCl in a covered test-tube by immersion in boiling water for 15 minutes. After
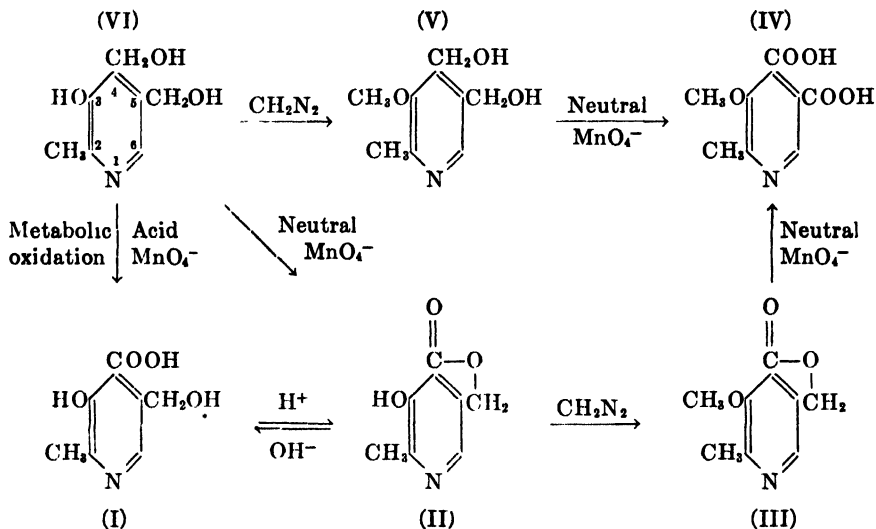
cooling, the urine is diluted to a known volume in order to bring the reading within the range of the instrument. The solution in the cuvette is brought to pH 9 by the addition of solid sodium borate. The blank reading is obtained from a sample of unheated urine in the same dilution and at the same pH. With the instrument used in this study a convenient standard consisted of a solution containing 0.04 γ of the lactone (II) per ml. of about 1 per cent sodium borate solution. The standard is not stable at this pH and is made up as required from a stock solution containing 0.4 γ per ml. in 0.1 N HCl solution.

It is possible to measure quantitatively the fluorescent metabolite in urine as the lactone, after heating with acid, without partial isolation, because the effect of interfering pigments and of other fluorescing sub-

stances is virtually eliminated by the extent of the dilutions employed. Thus a 4 hour human urine collected after a 50 mg. dose of pyridoxine is diluted 500 to 1000 times for the fluorometric reading. A normal urine is diluted 100 to 200 times, depending on the volume. Quantitative data on the excretion of the metabolite under varying conditions will be given in a subsequent communication.

Isolation of Metabolite (I) from Urine—A 24 hour urine was obtained from a normal individual who had ingested 1.25 gm. of pyridoxine hydrochloride in 125 mg. portions hourly over a period of 10 hours. The urine was adjusted to pH 4.5 with acetic acid and passed through two 42 × 2.5 cm. columns of Decalso,[1] 60 to 80 mesh, previously washed with dilute
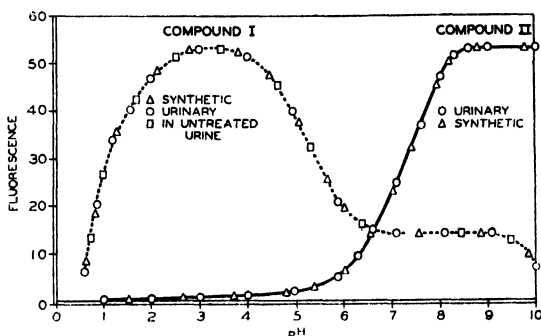
Fig. 1. pH-fluorescence curves   The ordinate represents the fluorescence in galvanometer divisions on the model 12 Coleman electronic photofluorometer. The broken line curve on the left is for aqueous solutions of compound (I) containing 1 0 γ per ml ; the urinary and synthetic compounds and also a sample of untreated urine are indicated by distinctive marks as shown   The solid line curve on the right is for aqueous solutions of compound (II), containing 0 04 γ per ml.; the urinary and synthetic compounds are indicated by distinctive marks

acetic acid, 25 per cent KCl, and water until free from Cl⁻. The effectiveness of adsorption was checked by fluorescence measurements. When the zeolite was saturated in respect to the compound, the latter was eluted with neutral 25 per cent KCl, the columns washed, and the adsorption and elution processes repeated twice. The KCl eluates were evaporated almost to dryness in an air current on the steam bath. The large crystalline mass of KCl was repeatedly extracted with small amounts of warm 95 per cent ethanol; the ethanol was evaporated and the salt residue reextracted with small volumes of 95 per cent ethanol. This procedure was repeated several times until the final extract in 20 ml. of ethanol was free of the greater portion of the salt. This alcoholic solution which contained about 30 mg.

[1] A zeolite obtained from The Permutit Company, 330 West 42nd Street, New York.

of compound (I) was evaporated to dryness and the residue dissolved in 10 ml. of hot water. After the solution was cooled in an ice bath, the dark precipitate which appeared was separated by centrifugation, the supernatant fluid discarded, and the sediment extracted three times with 1.5 ml. portions of 0.1 N KOH. The combined alkaline extracts were acidified with a small excess of 5 N HCl, at which point the compound precipitated in poorly defined crystalline form, which was filtered off. The precipitate was washed on the filter with small portions of cold water and 22 mg. of light brown material were obtained. Two recrystallizations from 3 ml. of hot pyridine yielded 10 mg. of a white crystalline product, m.p. 247–248° (uncorrected, in a bath previously heated to 200°).

Isolation of Lactone Form (II) of Metabolite from Urine—A 20 hour urine was collected from a normal individual who had ingested 1.0 gm. of vitamin B₆ hydrochloride in 100 mg. portions over a period of 10 hours. The urine was rendered 1 N acid with H_2SO_4 and heated in a boiling water bath for ½ hour. This treatment converts the metabolite (I) into its lactone form (II). A fluorometric measurement at this point indicated that there were 288 mg. of the lactone present. After being cooled and neutralized to pH 5 to 6, the urine was evaporated to a mushy residue of about 60 ml. on the water bath in a current of air. The residue was extracted with two 100 ml. portions of 95 per cent ethanol. The alcoholic extracts were evaporated almost to dryness and taken up in water to a volume of 40 ml. This dark solution was adjusted to pH 6.5, saturated with solid Na_2SO_4, and placed in a continuous ether extractor for 48 hours. The ether extract at the end of this time contained 222 mg. of the 288 mg. of lactone estimated to be present in the original urine. The ether extract was evaporated to dryness and the residue washed twice with 5 ml. portions of cold (−5°) absolute ethanol to remove pigments. The washed residue was taken up in 23 ml. of 95 per cent ethanol, 0.5 volume of ether added, and the solution cooled in ice water. The resulting precipitate was washed once with 5 ml. of alcohol-ether mixture (2:1) and discarded. The alcohol-ether filtrate and washing were heated to remove the ether. The alcohol solution was then concentrated until crystallization began. At this point the solution was immediately placed in an ice-cold bath. After 1 hour, 170 mg. of light brown crystals were filtered off and washed with cold absolute ethanol. By rapid crystallization adsorption of urine pigments on the crystals was avoided. After three recrystallizations from 95 per cent ethanol, 72 mg. of the crystalline lactone were obtained, in long prisms, m.p. 263–265° (uncorrected, in a bath previously heated to 200°).

Analysis[2]—$C_8H_7O_3N$.   Calculated.   C 58.18, H 4.24, N 8.48
                           Found.   " 58.10, " 4.38, " 8.69

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[2] The elementary analyses reported in this paper were carried out in the Laboratory of Microchemistry, Dr. Carl Tiedcke, New York.

Synthesis of Lactone Form (II) of Metabolite from Pyridoxine—To a solution of 600 mg. of vitamin B$_6$ hydrochloride (Merck) in 20 ml. of water, there were added with mechanical stirring 45.5 ml. of 0.5 N barium permanganate[3] at the rate of 0.3 ml. per minute. As indicated by a fluorometric measurement, this treatment produced 319 mg. of compound (II). The yield of the lactone is greatly reduced if the oxidation is carried out at a faster rate or more permanganate than indicated above is used. At the end of the oxidation, the MnO$_2$ was centrifuged off and washed five times with 5 ml portions of hot water. The filtrate and washings which were at pH 6.2 were concentrated to about 15 to 20 ml. under reduced pressure and adjusted to pH 6.4. The solution was saturated with Na$_2$SO$_4$ and subjected to continuous ether extraction for 48 hours. The ether extract by fluorometric measurement contained 300 mg. of the original 319 mg. estimated to be present after the oxidation. The ether extract was evaporated to dryness and the yellow residue dissolved in 35 ml. of hot (65°) 95 per cent ethanol, filtered, and the filtrate concentrated until crystallization began. The solution was immediately placed in an ice bath. After 1 hour, 240 mg. of light yellow crystals were filtered off and washed with cold absolute ethanol After two recrystallizations from 95 per cent ethanol, 155 mg. of the lactone (II) were obtained, m.p. 263–265° (uncorrected, in a bath previously heated to 200°); the mixed melting point with the urinary lactone was 263–265°. The crystals are well defined long prisms, as shown in Fig. 2, *b.*

Analysis[2]– C$_8$H$_7$O$_3$N   Calculated.   C 58 18, H 4.24, N 8 48
                        Found   " 57 87, " 4 47, " 8.57

Preparation of Compound (I) from Synthetic Lactone (II)—25 mg. of the synthetic lactone (II), prepared as above, in 3 ml. of 0.1 N KOH were gently boiled in a small beaker over a free flame for 12 minutes until the volume was reduced to about 0.5 ml. and cooled. A few drops of 5 N HCl were added to strong acidity. The compound precipitated out rapidly in a white mass and was filtered and washed free of salts and acid with ice-cold water. The product was recrystallized three times from 3 ml. of boiling pyridine to give 10 mg. of white whetstone and wedge-shaped crystals (Fig. 2, *a*), m.p. 247–248° (uncorrected, in a bath previously heated to 200°). This compound (I) may also be obtained by the direct oxidation of pyridoxine in a strongly acid (0.5 N HCl) solution with permanganate.

[3] Presumably KMnO$_4$ can be used. We employed the barium salt with the original intention of removing the cation later. This proved unnecessary.

However, under these conditions there are also produced small amounts of the lactone, and the separation of the two forms presents difficulties.

The identification of the urinary with the synthetic products is based upon the melting points, quantitative fluorescence, and elementary analyses, summarized in Table I. In addition, the identity is further demonstrated by the coincidence of the pH-fluorescence curves shown in Fig. 1 and also by the similar behavior in regard to the reversible reaction,

$$\text{Compound (I)} \xrightleftharpoons[\text{OH}^-]{\text{H}^+} \text{compound (II)}$$
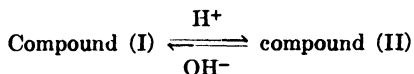
Properties and Structure of Metabolite—The metabolite is a white crystalline solid having a melting point of 247–248° (uncorrected). It is slightly soluble in water, alcohol, and pyridine. It is insoluble in ether and in

FIG. 2, a   FIG. 2, b

FIG 2   (a) Crystals of compound (I), (b) crystals of compound (II). × 790

aqueous acid solution but completely soluble in alkaline solution. It possesses two acidic groups, one a phenolic group and the other a carboxyl having pK values of 9.75 and 5 50 respectively, as is seen in the titration curve in Fig. 3. In aqueous solution under ultraviolet light it exhibits a characteristic blue fluorescence, the intensity of which follows a characteristic curve when plotted against the pH, as seen in Fig 1, reaching a maximum at pH 3 to 4. The fluorescence disappears on reduction with hydrosulfite and is restored to the original intensity by oxidation with H_2O_2. The metabolite is adsorbed on zeolite from aqueous solutions at pH 4 to 5 and is eluted with 25 per cent KCl; n-butanol extracts of the neutral eluates yield the same characteristic blue fluorescence as was observed in aqueous solutions. The extraction into butanol is maximal at pH 6 to 7. Singal and Sydenstricker (1) originally observed a fluores-

cence appearing in isobutanol extracts of urine collected after doses of pyridoxine. These investigators apparently determined the minimal fluorescence in neutral butanol solution. However, the intensity is increased to a maximum if the butanol is acidified with a few drops of glacial

TABLE I

Comparison of Synthetic and Urinary Oxidation Products, Compounds (I) and (II) of Pyridoxine

	M p. of acid, compound (I)	M p of lactone (II)	M p. of methyl ether (III)	Fluorescence*		Analysis of lactone (II)		
				(I)	(II)	C	H	N
	°C.	°C	°C.	*per cent*	*per cent*	*per cent*		
Synthetic .	247–248	263–265	111–112	51	1300	57.87	4.47	8.57
Urinary .	247–248	263–265	111–112	51	1300	58.10	4.38	8 69
Mixed m.p.	247–248	263–265	111- 112			58.18†	4.24†	8.48†

* The fluorescence is expressed in galvanometer divisions per microgram per ml. in the Coleman electronic photofluorometer with Filters B1 and PC1.

† Calculated.
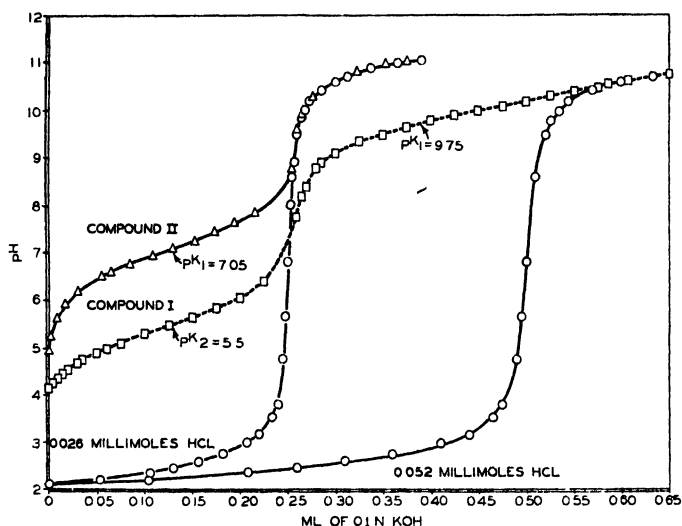
FIG. 3. Titration curves of 0.026 mM of compounds (I) and (II). For comparison the titration of 0 026 and 0.052 mM of HCl are also given.

acetic acid. The metabolite (I) is stable to boiling with dilute alkali (1 N), but upon being heated with 0.5 N acid for 10 minutes is converted to compound (II), possessing also a blue fluorescence. In this case, however, the maximal intensity is at pH 8.6 to 10 compared to pH 3 to 4 for the original compound. The fluorescence intensity of compound (II) at its maximum

is 25 times greater than that of compound (I) at its respective maximum, and follows a well defined pH-fluorescence curve, as illustrated in Fig. 1. Compound (II) possesses one weakly acidic group with a pK value of 7.05, as shown in the titration curve in Fig. 3. This compound when heated with alkali is reconverted into compound (I) with the neutralization of 1 equivalent of alkali.

The *structure* of the metabolite (I) was determined by way of compound (II), since the latter form offered several distinct working advantages over the former. The lactone (II) is more readily obtainable from urine, it possesses greater solubility in water, and it exhibits a far greater fluorescence intensity; thus the fate of the compound could be more readily followed quantitatively in the course of chemical reactions.

An elementary analysis of compound (II) indicates an empirical formula of $C_8H_7O_3N$, which corresponds to the lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine. The molecular weight of 165 was confirmed by titration (Fig. 3).

Heating an aqueous solution of compound (II) with CNBr at 75° for 5 minutes, cooling, and adding saturated aqueous aniline gave the characteristic yellow color produced by pyridine and its derivatives. This indicates the presence of pyridine nitrogen in its uncombined, tertiary state.

Treatment of an aqueous solution of compound (II) with $FeSO_4$ gave no color reaction; thus the compound contained no α-COOH group in position 2 (3). α-Picolinic acid under similar conditions gave a strong color reaction. Since compound (II) can be obtained by permanganate oxidation of pyridoxine which contains an α-methyl group, and since the $FeSO_4$ test was negative, indicating no —COOH group in this position in compound (II), it was concluded that the methyl group had remained intact during the oxidation.

That position 3 on the pyridine ring is occupied by a phenolic hydroxyl group is demonstrated by the following evidence. A potentiometric titration of the lactone (II) showed the presence of a weakly acidic group (pK_1 7.05, Fig. 3); a strong $FeCl_3$ reaction for the phenolic group was obtained; the compound yielded a methyl ether (m.p. 111–112°) upon treatment with diazomethane[4] which gave a negative test with $FeCl_3$. Treatment of an alkaline solution of compound (II) with Gibbs' 2,6-dichloroquinonechloroimide reagent (5) gave the characteristic blue color. A positive color reaction was obtained when compound (II) was coupled with diazotized p-bromoaniline, indicating a β-hydroxy group (6).

Since compound (II) has a hydroxyl group in position 3, then position 6 on the ring remains unsubstituted, as indicated by the positive color reaction with the 2,6-dichloroquinonechloroimide reagent. Gibbs (5) has shown that p-substituted phenols do not react with this reagent.

[4] The methyl ether was synthesized by a method described by Harris *et al.* (4).

The evidence presented indicates that the portion of the structure of compound (II), except for the groups occupying positions 4 and 5, is identical with that of pyridoxine itself. The following experiment demonstrates conclusively that this is the case. Treatment of the lactone (II) with the diazomethane[4] yielded its methyl ether (III), which, upon oxidation with neutral $Ba(MnO_4)_2$, was converted into a dicarboxylic acid.[5] The melting point was 207–208°, although it varied, depending upon the rate of heating. This compound gave a negative test for an α-COOH group (3) with ferrous sulfate, and a negative reaction for a phenolic group with ferric chloride. Titration of the compound with alkali resulted in the consumption of 2 equivalents. The compound was heated at 130° with resorcinol and a few drops of concentrated H_2SO_4 for a few minutes. When the mixture was made alkaline, a phthalein having a greenish yellow fluorescence was produced, indicating that the two carboxyl groups are on adjacent carbon atoms (7). 2-Methyl-3-methoxy-4,5-dicarboxypyridine (IV) was prepared according to the method of Stiller *et al.* (6). The melting point of this compound (IV) was 205–206°, which also varied, depending upon rate of heating. The mixed melting point of the dicarboxylic acid (IV) and the dicarboxylic acid as prepared above from the lactone (II) was 205–206°. The dicarboxylic acid derivative of the lactone (II) is therefore 2-methyl-3-methoxy-4,5-dicarboxypyridine (IV) and thus proves that the portion of the structure of the lactone (II), other than the groups in positions 4 and 5, is identical with that of pyridoxine.

This leaves the two groups in positions 4 and 5 for consideration. The fact that compound (II) is an oxidation product of pyridoxine which contains —CH_2OH groups in positions 4 and 5 simplified the problem somewhat.

The following reasoning is presented to show that positions 4 and 5 in the metabolite (I) are occupied by a —COOH and a —CH_2OH respectively, and that in compound (II) a lactone ring exists between positions 4 and 5. When compound (II) was heated with alkali, 1 equivalent was neutralized with the liberation of one —COOH group, as shown in the titration curve, Fig. 3. Scudi and collaborators (8) utilized the different behavior of the chloroimide indophenol reaction in the presence of veronal and borate buffers in the elucidation of the structure of several metabolites of pyridoxine. In this study it was found that compound (II) gave a positive reaction in the presence of both buffers, whereas compound (I) gave a positive reaction with veronal and a negative reaction with the borate buffer.

These data indicate that position 4 is occupied by a —CH_2OH group and position 5 by a —COOH group or vice versa, and that in compound (II) the

[5] The dicarboxylic acid was synthesized by a method described by Harris *et al.* (4).

group in position 4 is not free to form a boron complex. The group in position 4 is apparently closed in a lactone ring with the group in the adjacent 5 position, as shown by the saponification reaction with alkali as described above. The lactone (II) then has one of two possible structures (II, IIa). It is either the lactone of a 4-hydroxymethyl-5-carboxy-

(II)   (IIa)
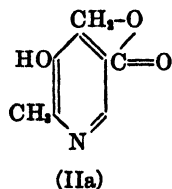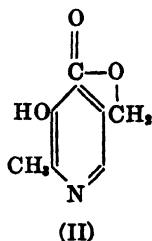
pyridine derivative (IIa) or a 4-carboxy-5-hydroxymethylpyridine derivative (II). The lactone (II) obtained from acid heated urine was compared as to melting point and quantitative fluorescence measurement with a sample of the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine (IIa)[6] with the following results. Although the individual melting points of the two lactones were identical (263–265°, with decomposition), the mixed melting point was depressed to 225°. The fluorescence, though qualitatively similar, was considerably different quantitatively. It is thus apparent that the urinary lactone (II) is not a 4-hydroxymethyl-5-carboxypyridine derivative (IIa) but is the isomer, a 4-carboxy-5-hydroxymethylpyridine derivative (II).

It is, therefore,· deduced that compound (II) has the structure given in the preceding set of formulas and that the free acid compound (I) obtained from it by heating with alkali represents the structure of the metabolite occurring in human urine.

That the oxidation product of pyridoxine is excreted in human urine in the form of the free acid (I) and not as a compound conjugated through the phenolic on hydroxymethyl group is shown by the exact coincidence of the pH-fluorescence curve (Fig. 1) measured in an untreated concentrated urine sample with that obtained from the crystalline compound isolated from the urine as above. Furthermore, when this urine was heated with acid, the increase in fluorescence, now measured at pH above 8.5, corresponded quantitatively with that observed on conversion of the synthetic acid (I) to the lactone (II).

The occurrence of the oxidative metabolite (I) in the urine of men and

[6] The sample was furnished through the kindness of Dr. K. Folkers of the Research Laboratories of Merck and Company, Inc. The compound was synthesized by Harris, Stiller, and Folkers (4) in their study on the structure of pyridoxine

of other species, as well as its relation to the other metabolite derivatives of pyridoxine, as described by Scudi and collaborators (8), will be discussed in a succeeding article.

SUMMARY

A fluorescent compound appearing in urine after the ingestion of pyridoxine was isolated and identified as 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (4-pyridoxic acid). Heating with mineral acid converts this compound into its lactone. The lactone was prepared synthetically from pyridoxine by oxidation with permanganate, and converted to the corresponding acid by heating with alkali.

The metabolite and the lactone possess characteristic pH-fluorescence curves. Based upon this property, a general fluorometric method for the quantitative determination of either compound was developed. The application of the method to the direct estimation of the lactone in the urine is described.

Addendum—After the completion of the above work the possible relation of the oxidative metabolite to the metabolite described by Snell, Guirard, and Williams (9) was clarified in the following manner.

Samples of the acid (I) and of the lactone (II) were sent by us to Dr. E. E Snell and were kindly tested by him for "vitamin B_6" activity employing *Streptococcus lactis* R, *Lactobacillus casei*, and yeast The results were as follows: The acid (I) showed almost no activity with any of these organisms. A sample of the lactone (II) prepared from the acid (I) used above was 0.24 as active as pyridoxine hydrochloride for *Streptococcus lactis*, 0 02 as active for *Lactobacillus casei*, and 0 00025 as active for yeast. Barring the possible production of small amounts of an active impurity during lactonization, the lactone (II) possesses only a very slight pseudopyridoxine activity. It is thus apparent that neither the acid (I) nor its lactone is identical with "pseudopyridoxine" as described by Snell and his collaborators An aldehyde and an amine of pyridoxine, as recently synthesized by Harris, Heyl, and Folkers (10), were shown to possess pseudopyridoxine activity in microbiological assays by Snell (11)

BIBLIOGRAPHY

1. Singal, S A , and Sydenstricker, V. P., *Science*, **78,** 545 (1941).
2. Huff, J. W , and Perlzweig, W. A., *Science*, **100,** 15 (1944).
3. Skraup, Z. H., *Monatsh. Chem.*, **7,** 210 (1886); cited in Maier-Bode, H., and Altpeter, J., Das Pyridin und seine Derivate, Halle-on-the-Saale, 212 (1934).
4 Harris, S A., Stiller, E. T., and Folkers, K., *J. Am. Chem. Soc* , **61,** 1242 (1939).
5. Gibbs, H. D., *J. Biol Chem.*, **72,** 649 (1927).
6. Stiller, E. T , Keresztesy, J C , and Stevens, J. R., *J. Am. Chem. Soc.*, **61,** 1237 (1939).
7. Bernthsen, A., and Mettegang, H., *Ber. chem Ges.*, **20,** 1208 (1887); cited in Maier-Bode and Altpeter (3), p. 231.
8 Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, **142,** 323 (1942).
9. Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143,** 519 (1942).
10. Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, **154,** 315 (1944).
11. Snell, E. E., *J. Biol. Chem.*, **154,** 313 (1944).

LETTERS TO THE EDITORS

A FUNCTION OF PYRIDOXAL

Sirs:

Previous work has shown that the rate of tyrosine decarboxylation by cell suspensions of *Streptococcus faecalis* is influenced by the concentration of pyridoxine added to the growth medium.[1] It has been found that auto-claving pyridoxine with cystine[2] or treatment with dilute hydrogen perox-ide[3] enhances its growth-promoting activity for lactic acid bacteria, and both of these preparations have been shown to stimulate the rate of tyro-sine decarboxylation by cell suspensions harvested from media deficient in pyridoxine or its derivatives.[4] The present report deals with the activity of two synthetic compounds, pyridoxal and pyridoxamine,[5] which were kindly supplied to us by Dr. E. E. Snell.

Pyridoxal under the conditions used stimulates the tyrosine decarboxy-lase system of *S. faecalis* R, whereas pyridoxamine does not.

Tyrosine decarboxylation was followed manometrically by determining the rate of CO_2 evolution. Each Warburg cup contained 0.5 ml. of $M/30$ tyrosine suspension, 1 ml. of 0.075 M phthalate buffer, pH 5.0, and water or test substance to make 2.5 ml. The side arm contained 0.5 ml. of cell suspension ($\backsim$ 0.24 mg. of bacterial nitrogen) in saline. The cells were harvested from the hydrolyzed gelatin medium,[1] which contains about 100 mg. per cent of alanine and thus supports the growth of the R strain of *S. faecalis* without added pyridoxine.[6]

The $Q_{CO_2}N$ of 415 is approximately the maximum value which has been obtained with this culture; other enterococci studied have shown values as high as 2500. The stimulation of tyrosine decarboxylation occurred after the first 10 minute interval and the linear rate was maintained for at least 2 hours.

[1] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **46**, 573 (1943); *J. Bact.*, in press.

[2] Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **51**, 356 (1942).

[3] Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc. Exp. Biol. and Med.*, **54**, 123 (1943).

[4] Gunsalus, I. C., and Bellamy, W. D., *J. Bact.*, **47**, 413 (1944); *J Biol. Chem.*, in press.

[5] Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).

[6] Snell, E. E., and Guirard, B. E., *Proc. Nat. Acad. Sc.*, **29**, 66 (1943).

Tyrosine Decarboxylation by Streptococcus faecalis R*

Pyridoxal	CO₂ evolved	$Q_{CO_2}N$
γ per 3 ml.	microliters per hr.	
0.0	5	20
0.02	12	50
0.04	23	96
· 0.06	32	137
0.1	45	188
0.3	85	355
0.6	90	375
3.0	100	415

* A. T. C No. 8043 under the name *Streptococcus lactis* R.

The dissociation constant of the pyridoxal-tyrosine decarboxylase enzyme may be approximated from the above data if one makes the usual assumptions that only the combined form is catalytic, and that the amount of combined pyridoxal is small in proportion to the total amount present. The dissociation constant

$$K = \frac{(\text{pyridoxal})(\text{uncombined protein})}{(\text{pyridoxal-protein})} \quad \text{or} \quad = \frac{(c)(V_{max} - v)}{v}$$

where $V_{max.}$ is the maximum reaction velocity of the system studied and c and v are respectively the concentration of pyridoxal and the corresponding rate of CO_2 evolution from the accompanying table. When the equation for the dissociation constant is rearranged in one of the linear forms,[7] as $1/v = 1/c\ K/V_{max.} + 1/V_{max.}$ and the data plotted, one obtains a $V_{max.}$ of 105 and a $K_{(\gamma\ per\ 3\ ml.)}$ of 0.15. As the molecular weight of pyridoxal is 168, the dissociation constant K is $0.15/(3 \times 1000 \times 168) = 3 \times 10^{-7}$ mole per liter. The relatively low dissociation constant indicates that only a small amount of pyridoxal should be required in cells for optimum function of this system.

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[7] Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).

THE STRUCTURE OF ASPERGILLIC ACID

Sirs:

Aspergillic acid is an antibiotic agent which was isolated by White and Hill[1,2] from a strain of *Aspergillus flavus*. Though the production and antibacterial properties of this substance have been studied in detail,[2,3] only a few chemical data have so far been recorded.[4] In the present communication we briefly present the results of further chemical work, and our conclusions regarding the structure of this compound.

Aspergillic acid, m.p. 93°, $C_{12}H_{20}O_2N_2$, is a monobasic acid (pK 5.5), which also possesses a weakly basic group (hydrochloride, m.p. 178°). It forms a highly characteristic grass-green cupric salt melting at 198°. A methanolic solution yields a deep red coloration on the addition of a drop of ferric chloride solution. That these properties are associated with an acidic hydroxyl group was shown by the conversion of aspergillic acid, either by dry distillation in the presence of copper chromite catalyst or by reduction with hydrazine, into an essentially neutral desoxy compound, $C_{12}H_{20}ON_2$, (m.p. 100°). Since the above reactions are typical for hydroxamic acids, the presence of the grouping —N(OH)·CO— was suspected.

Both aspergillic acid and the desoxy compound show a well defined single maximum at 325 mμ (ϵ = 8500) in their ultraviolet absorption spectrum. They are both optically active ([α]$_D$ = +13° and +10°, respectively, in ethanol). They exhibit remarkable stability towards acidic and alkaline hydrolytic agents and are indifferent to neutral potassium permanganate solution. These properties, coupled with the additional facts cited later, suggested the presence of an α-pyrazone nucleus, with substituent alkyl groups carrying the asymmetric carbon atom (or atoms). With the aid of biogenetic considerations the accompanying structures for aspergillic acid (I) and desoxyaspergillic acid (II) were formulated.

These formulas receive support from the following findings: (1) The ultraviolet absorption spectrum of α-pyrazone exhibited a maximum at 315 mμ (ϵ = 4500). (2) Aspergillic acid, as well as the desoxy compound, is reduced by zinc in acetic acid to a tetrahydrodesoxy derivative, $C_{12}H_{24}ON_2$, isolated as the hydrochloride (m.p. 260°). (3) Aspergillic acid can be brominated in aqueous acid media to a monobromoaspergillic acid, $C_{12}H_{19}O_2N_2Br$, (m.p. 129°) which still exhibits the typical reactions for hydroxamic acids. Similarly, desoxyaspergillic acid yields a mono-bromo derivative $C_{12}H_{19}ON_2Br$ (m.p. 130°). The alkali stability of these

[1] White, E. C., *Science*, **92**, 127 (1940).
[2] White, E. C., and Hill, J. H., *J. Bact.*, **45**, 433 (1943).
[3] Jones, H., Rake, G., and Hamre, D., *J. Bact.*, **45**, 461 (1943).
[4] Menzel, A. E. O., Wintersteiner, O., and Rake, G., *J. Bact.*, **46**, 109 (1943).

(I)　　　　　　　　　　　　　　　　　　　(II)

(III)
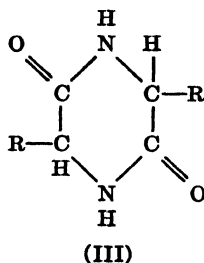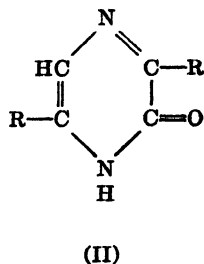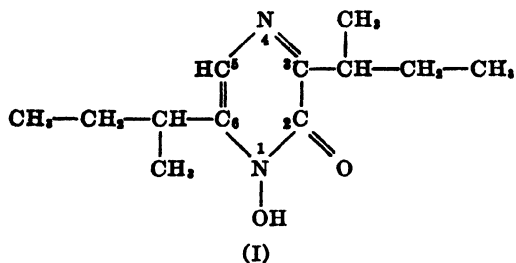
bromo compounds indicates substitution in the nucleus; *i.e.*, in the 5-position. (4) In 50 per cent acid solution desoxyaspergillic acid is oxidized by bromine to a quinone-like compound ($C_{12}H_{18}O_2N_2$, m.p. 270°, ultraviolet maximum at 285 mμ, ϵ = 22,200). The latter on catalytic hydrogenation absorbs 2 moles of hydrogen and is thereby transformed into a neutral, optically active substance, $C_{12}H_{22}O_2N_2$ (m.p. 250–252°, with sublimation), which possesses the typical properties of a diketopiperazine. Racemic isoleucine anhydride prepared from *dl*-isoleucine melted at 257° and was indistinguishable from Compound III in appearance and solubility properties. Conclusive identification is rendered difficult because (III) may represent only one of the four stereoisomers of isoleucine anhydride. Unfortunately this diketopiperazine is extremely resistant to hydrolysis.[5] However, further structural evidence was adduced by the reduction of aspergillic acid with sodium and amyl alcohol to an optically active piperazine base, $C_{12}H_{26}N_2$ (nitrate, m.p. 222°). The corresponding racemic base was obtained by the same reaction from synthetic isoleucine anhydride. Comparison of several derivatives (picrate, nitrate, dibenzoate, nitroso compound) left little doubt as to the structural identity of the two compounds.

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⁵ Ehrlich, F., *Ber. chem. Ges.*, **40**, 2550 (1907).

FOLIC ACID IN COTTON

Sirs:

In the determination of folic acid by means of the metabolism of *Lactobacillus casei* ε, it was observed that duplicate tubes frequently produced widely varying amounts of acid. The extraction of inhibitory or stimulatory substances from the cotton plugs was suspected. An aqueous extract of the cotton used for plugging the tubes was tested together with suitable controls, and full growth of *L. casei* ε as measured by acid production was obtained in tubes which contained little or no added folic acid.[1] Assays on aqueous extracts of weighed amounts of cotton indicated that two samples of absorbent cotton contained less than 0.00027 γ per gm., while the third sample (non-absorbent cotton) contained at least 100 times as much of the growth activator. The non-absorbent cotton used showed particles which appeared to be fragments of bolls or seeds. Hence intact bolls[2] of Upland cotton were obtained; the fiber, seeds, and bolls were separated, and ground, weighed, and extracted with 20 volumes of boiled distilled water for 2 hours at 37° Assays of these extracts indicated that the fiber contained approximately 0.028 γ per gm., the bolls approximately 0.5 γ per gm., and the seeds 0.75 γ per gm. Extracts of seeds obtained from other sources[3],[4] were remarkably uniform, containing 0.8 to 1.2 γ of active substance per gm. of raw material.

That the growth-stimulatory substance in the crude extracts is folic acid is attested by the following data. (1) It stimulates the growth of both *Streptococcus lactis* R and *Lactobacillus casei* ε in folic acid-free media. (2) It is water-soluble, but relatively insoluble, at least in the crude state, in ethyl ether, ethyl alcohol, acetone, benzene, chloroform, and pyridine. (3) It is destroyed by methods known to destroy folic acid.

Acid hydrolysis (autoclaving in 1 N sulfuric acid) destroyed 99 per cent.

Treatment with glacial acetic acid failed to destroy the material, but reduction with zinc in the presence of glacial acetic acid destroyed 37 per cent.

Oxidation with 1 per cent hydrogen peroxide destroyed 34 per cent activity.

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[1] We are indebted to Dr. R. J. Williams, The University of Texas, for the sample of folic acid used.

[2] The bolls were received from Arthur Mosby, Memphis, Tennessee, through the kindness of the Lockport Cotton Batting Company

[3] T. L. Carter, Sr., Tutwiler, Mississippi.

[4] Lockport Cotton Batting Company, Lockport, New York

Further experiments dealing with the age of the cotton plant, the source of the material in relation to the folic acid content, and simple methods of concentration are in progress.

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ON THE ENZYMATIC SPECIFICITY OF RENIN

I. THE PROTEINASE COMPONENTS OF RENIN PREPARATIONS AND THEIR RELATION TO RENIN ACTIVITY

BY ALBERT A. PLENTL AND IRVINE H. PAGE

(From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis)

It has frequently been suggested (1, 2) that renin is a proteolytic enzyme, and that its action is the selective hydrolysis of one or more peptide linkages of the globulin renin substrate. The observation of Croxatto and Croxatto (3) that pepsin can be substituted for renin in the preparation of an angiotonin-like pressor substance which they called pepsitensin led Croxatto, Croxatto, and Alliende (4) to the conclusion that renin belongs to a group of enzymes (carbonylproteinase) of which the best known representative is pepsin. In view of the fact that they used a number of synthetic substrates which are generally employed for enzyme studies, it must be assumed that their suggestion is meant to imply homospecificity of pepsin and renin. The meaning of the term homospecificity will become clear from the following, although somewhat brief, definition of the word.

A peptide linkage is attacked by a particular proteolytic enzyme if it occurs in the proper environment of amino acid residues. Two substrates which differ only with respect to 1 of their amino acid residues but are identical in all other structural details will be hydrolyzed by an enzyme at different rates, as expressed in terms of the proteolytic coefficients. If the ratio of these coefficients, the *proteolytic quotient,* is identical for both enzymes under consideration, the enzymes are "homospecific." If, on the other hand, the numerical value of the quotient for one enzyme is different from the one obtained by allowing another enzyme to act on the same pair of substrates, this pair of enzymes is "heterospecific."

Since the criteria of chemical purity cannot be met for most intracellular proteolytic enzymes, Bergmann and his associates characterized the specificity of these proteinases by comparing the rates at which well chosen synthetic substrates are hydrolyzed by them (5). They thus arrived at a new system for the classification of proteolytic enzymes which appears to be the most clear cut yet devised (6). It is the object of this investigation to determine whether renin is to be placed in any of the general groups of intracellular enzymes as defined by Bergmann and his associates.

Methods and Materials

The enzyme solutions employed were prepared according to the procedure described by Plentl and Page (7). This procedure consists of saline extraction of defatted and dried kidney powder followed by two precipitations with sodium chloride at pH 4.0. On repetition of the sodium chloride precipitation the resulting renin solutions were found to be practically free of angiotonase. Such solutions were frozen and dried in the frozen state. The residual light, dry powder could be stored at room temperature for months without loss of activity. When needed, it was dissolved in normal saline or distilled water to yield the desired concentration, the nitrogen content being determined by the micro-Kjeldahl method.

For storage or long incubation experiments all solutions were kept sterile and for short term experiments merthiolate (sodium ethylmercurithiosalicylate, Lilly) was added in such quantities as seemed necessary to prevent bacterial growth.

Crystalline Pepsin—This was prepared according to the procedure of Northrop (8). For all experiments involving biological assays, the enzyme was dissolved in normal saline and the nitrogen concentration determined by the micro-Kjeldahl method.

The synthetic polypeptides which were used in this investigation were prepared according to the various procedures reported by Bergmann and his associates (Table I). The course of the hydrolysis was followed by increase in amino nitrogen as determined by the method of Van Slyke except in the case of leucinamide in which the increase of free amino acids was determined by the method of Van Slyke, Dillon, MacFadyen, and Hamilton (14). Our first order reaction constants were calculated according to the formula $K = 1/t \log a/(a - x)$. The proteolytic coefficients were then calculated by dividing K by the mg. of protein nitrogen per cc. of the test solution (15).

The pressor strength of angiotonin or pepsitensin solutions was determined by bioassay, pithed cats being used as test animals. Reaction constants and proteolytic coefficients were calculated as described above.

The proteolytic coefficient, C, referring to a specific substrate, X, and the enzyme, Y, is written as C_X^Y. In order to simplify the writing of proteolytic coefficients, the following abbreviations are used throughout this paper: P for pepsin, PT for pepsitensin, R for renin, RS for renin substrate, SKCP for swine kidney carboxypeptidase, and PCP for pancreatic carboxypeptidase. The synthetic substrates were designated by the usual abbreviations (6).

The determination of renin was carried out as previously described (7). Rather than use "renin units" we preferred the proteolytic coefficient,

C_{RS}, to express renin activity. Since "renin units" by definition are proportional to the reaction constant, C_{RS} was calculated from the equation.

$$C_{RS} = \frac{(\text{renin units}) \times 10^{-2}}{\text{mg. protein N per cc. test solution}}$$

TABLE I

Effect of Crude Renin Preparations on a Number of Typical Synthetic Substrates

Substrate*	Temperature	N per cc. test solution	K†	C‡	Enzyme indicated by hydrolysis
	°C	mg			
Carbobenzoxyglycyl-*l*-phenylalanine (9)	37	0 17	0.0022	0 013	Carboxypeptidase
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine (10)	30	0.11	0 0011	0.010	Pepsinase
Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine (9)	30	0 11	0.00079	0 0072	"
l-Leucinamide§ (11)	37	0.39	0.0066	0 017	Aminopolypeptidase (leucylpeptidase)
l-Leucylglycine (12)	37	0 77	0 0027	0 035	" "
Benzoyl-*l*-argininamide (13)	37	Hydrolyzed			Trypsinase

The pH of all incubation mixtures was maintained at 5 3 by means of 0 1 N citrate buffer

* The figures in parentheses represent the bibliographic references in which the preparation of the various substrates is described.

† $K = \dfrac{1}{t} \log \dfrac{a}{a-x}$.

‡ $C = \dfrac{K}{\text{mg. N per cc. test solution}}$.

§ Cysteine added.

DISCUSSION

The intracellular proteolytic enzymes of animal tissues are generally divided into four groups, which Bergmann originally designated as Cathepsins I, II, III, and IV (16)[1] and for which he has more recently (6) preferred the nomenclature pepsinases, trypsinases, aminopeptidases, and carboxypeptidases, because of their analogy to the enzymes of the gastrointestinal tract. If renin is to be regarded as an intracellular proteolytic enzyme, it might be identical or homospecific with any one of the cathepsins defined above or be in a group by itself.

If it is to be decided whether a certain enzyme activity of a particular preparation can be attributed to one or more principles in this preparation, it is customary in enzyme chemistry to demonstrate first the presence of

[1] Recently Rocha e Silva and Andrade defined Cathepsin V (17).

the enzymes in a qualitative way and then study the enzymatic homogeneity of the sample by means of activity ratios. Fractionation procedures which need not necessarily result in chemical purification of the substance but may include fractional denaturation are then applied and the activity ratios are compared to the original. If many different methods of fractionation fail to alter the activity ratio for any pair of supposed enzyme activities, the preparation is homogeneous in regard to these activities. If, for example, the ability of kidney extracts to produce angiotonin from blood proteins is to be attributed to a certain proteinase activity as measured by its ability to hydrolyze especial synthetic substrates, the ratio of proteinase to renin activity must not change no matter what fractionation or fractional inactivation procedure had been employed.

If, on the other hand, a certain preparation shows all of the necessary chemical criteria of purity, e.g. homogeneity in the ultracentrifuge and after electrophoresis as well as its phase rule relations, it can be assumed to be enzymatically homogeneous. We therefore attempted to prepare renin in relatively pure form before going any further in our studies of its enzymatic specificity.

Renin samples prepared according to the directions given in the literature are generally rather crude mixtures of intracellular proteins which do not even approach the criteria of chemical purity. In this connection, a recent paper by Katz and Goldblatt (18) on the purification of renin is of interest. They believed that they had prepared an electrophoretically homogeneous renin. An electrophoretic analysis, for which we are indebted to Dr. W. W. Davis and Mr. T. V. Parke, of a sample prepared according to their method indicated at least five, possibly seven different components. It seems probable that Katz and Goldblatt used too short an exposure to effect adequate resolution in the electric field. Their sample was stated to be homogeneous at 50 minutes exposure but no information is given beyond this time. Since the values of the mobilities of these protein constituents are very close together, at least 200 or 300 minutes exposure would be required for adequate separation.

Although admittedly very impure from a physicochemical point of view, our renin preparations seemed to lend themselves well for a study of their enzymatic characteristics. Since it would appear highly improbable that the samples are enzymatically homogeneous, a qualitative analysis for their enzyme constituents seemed indicated.

Several synthetic polypeptides were incubated with different amounts of renin samples and the degree of hydrolysis determined by the usual methods. The results of this study are given in Table I, from which it can be seen that the preparation contains all four common cathepsins. These include carboxypeptidases, pepsinases, aminopeptidases which might

include leucylpeptidases, and trypsinases. Carboxypeptidases hydrolyze carbobenzoxy-*l*-glutamyl-*l*-tyrosine and carbobenzoxyglycyl-*l*-tyrosine (19), while pepsinases hydrolyze the latter substrate only to an insignificant extent (20). The rate at which carbobenzoxy-*l*-glutamyl-*l*-tyrosine is hydrolyzed by carboxypeptidase is very much greater than the hydrolysis of this substrate by pepsin. Hence, the only conclusion that can be drawn from the finding that both carbobenzoxyglycyl-*l*-phenylalanine as well as carbobenzoxy-*l*-glutamyl-*l*-tyrosine are hydrolyzed by this renin preparation is that it contains kidney carboxypeptidase but not necessarily kidney pepsinase.

The hydrolysis of leucinamide and leucylglycine indicates the presence of aminopeptidase or leucylpeptidase. No hydrolysis occurs in the absence of cysteine, which would therefore rule out the suggestion that renin might be identical with this enzymatic constituent, for renin activity requires no activation. This identity appears even more unlikely if it is considered that an exopeptidase can hardly be responsible for the formation of a polypeptide (angiotonin) from a high molecular weight protein (renin substrate) unless the linkage of angiotonin to the residual renin substrate should be of such a nature that it becomes sensitive to hydrolysis by aminopeptidases. If this is true, then angiotonin must be formed from renin substrate by splitting off of 1 amino acid residue. Only an acid or basic amino acid can be considered, since it must have at least one functional group in addition
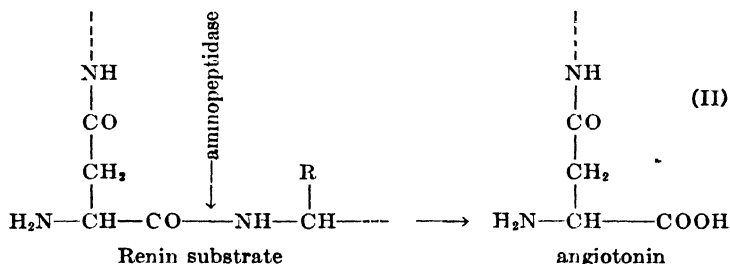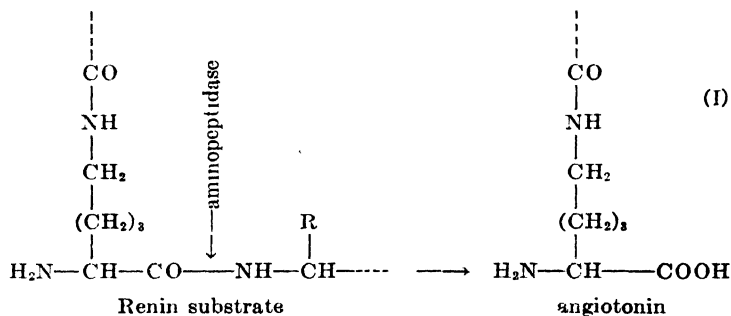
to its α-amino and carboxyl group. This functional group must then be involved in peptide linkage with a chain of amino acids. With lysine and aspartic acid as examples, this concept will become clear from Formulas I and II of this moiety of the renin substrate molecule.

In addition to the objections outlined above (no activation necessary) it must be pointed out that angiotonin is inactivated by aminopeptidase and carboxypeptidase. Formula I does not show a peptide linkage sensitive to aminopeptidase hydrolysis and a compound of Formula II would probably not be hydrolyzed by carboxypeptidase.

The hydrolysis of benzoylargininamide was not followed quantitatively because it must be determined by an increase in titratable carboxyl groups (13), a procedure which, in our hands, did not prove sufficiently accurate for the calculation of reaction constants. Since the rate of hydrolysis was not very fast, high concentrations of protein were required, which caused a large amount of undesirable precipitate during the titration and also masked the color change of the indicator. Suffice it to say that this kidney extract hydrolyzed benzoylargininamide to some extent, but the enzymatic component indicated by this hydrolysis cannot be identical with the enzyme, renin, for reasons discussed below.

The data reported in Table I can also be used for semiquantitative analysis. Since the proteolytic coefficient (C), i e. the reaction constant divided by the enzyme concentration (in mg. of N per cc.) represents a direct measure of activity, it also becomes a measure of purity for the enzyme preparation. The purest sample will give the highest proteolytic coefficient. Although the C values in Table I are not strictly comparable to similar values reported by other investigators because of some differences in incubation temperature, we can, nevertheless, conclude that, with the exception of leucyl- or aminopeptidase, none of the cathepsins indicated in the last column has been purified or its relative concentration increased when our swine kidney extracts are compared with those of Fruton, Irving, and Bergmann (16). They reported values of 0.034 for the hydrolysis of carbobenzoxyglycylphenylalanine (carboxypeptidase, 40°), 0.0032 for carbobenzoxyglutamyltyrosine (pepsinase, 25°), and 0.0017 for leucylglycine (aminopeptidases, 40°). Although our values for the hydrolysis of carbobenzoxyglycylphenylalanine by carboxypeptidase are not strictly comparable to those of Fruton, Irving, and Bergmann, since these authors used cysteine activation to enhance the activity of the enzyme, a decrease in pepsinase activity and an increase in aminopeptidase are indicated. Yet the renin activity of the sample was at its maximum.

The fact that the catheptic activity of our renin preparation (per mg. of N) had decreased, when compared to a crude saline extract of kidney, cannot be interpreted as evidence that renin differs in its action from all

known intracellular proteinases. The statement that renin activity had been increased by the purification procedure outlined in the experimental part must not be taken at its face value, for it refers to apparent activity only. If, for the sake of the argument, renin activity is attributed to pepsinase, and angiotonase activity to carboxypeptidase, it may well be that the original saline extract had so great a concentration of angiotonase (carboxypeptidase) that the renin (pepsinase) activity could not be demonstrated at all, but may have been present in very much greater concentration than is evident from the data in Table I. It is only when the ratio of angiotonase to renin activity is 1:5 or less that renin can be accurately determined by measurement of the first order reaction constant (7).

The fact that carboxypeptidase concentration is many times greater than the concentration of pepsinase is of little importance in reference to the aforementioned possible relation of renin and angiotonase, since the magnitude of C is dependent not only on the enzyme but also the substrate. Pepsinase hydrolyzes carbobenzoxy-l-glutamyl-l-tyrosine very slowly but may act on renin substrate sufficiently fast to overshadow the secondary action of carboxypeptidase on angiotonin.

Still another deduction can be made from the data in Table I. The most sensitive characteristic of such an enzyme seems to be the consistent ratio of proteolytic coefficients for two substrates which differ only with respect to 1 amino acid residue. Fruton, Irving, and Bergmann showed that pepsinases hydrolyze tyrosine-containing compounds approximately twice as fast as the corresponding phenylalanine substrates (16). This is obviously not true for crude renin samples such as the one employed for the experiments summarized in Table I. When it is recalled that carboxypeptidase hydrolyzes carbobenzoxy-l-glutamyl-l-phenylalanine faster than the tyrosine compound (16), it becomes clear that if both enzymes act simultaneously the ratio cannot be consistent for either enzyme.

As outlined above, the fact that carbobenzoxyglycyl-l-phenylalanine is hydrolyzed quite rapidly is sufficient evidence for the statement that carboxypeptidase is present, but for a qualitative demonstration of the presence of pepsinase the former enzyme must be removed entirely. Fortunately, this can be accomplished by thorough dialysis (16). A comparison of the enzymatic characteristics of the original sample and one which had been dialyzed for 72 hours against distilled water is given in Table II. Carboxypeptidase activity had decreased approximately 100 times, while the proteolytic coefficient for carbobenzoxy-l-glutamyl-l-tyrosine (which includes both carboxypeptidase and pepsinase activity) had decreased to about one-sixth of its former value. Since the proteolytic coefficient for the hydrolysis of carbobenzoxyglycyl-l-phenylalanine appears to be many times greater than the coefficient for carbobenzoxy-l-glutamyl-

l-tyrosine for carboxypeptidase action (19), it is difficult to escape the conclusion that the hydrolysis of the latter substrate by the dialyzed material is due almost exclusively to pepsinase.

It now remains to be shown that the dialyzed material is homogeneous with respect to pepsinase activity.   This becomes evident from the data

TABLE II

Elimination of Carboxypeptidase Component by Dialysis

	$C^* \times 10^3$	
	Preparation A	Preparation B
Carbobenzoxyglycyl-l-phenylalanine†	13 02	0.14
Carbobenzoxy-l-glutamyl-l-tyrosine‡	10 8	1.50

Preparation A was made according to the general directions reported in an earlier publication (7)   This was dialyzed for an additional 72 hours against distilled water, the euglobulins being removed by centrifugation   Preparation B represents the supernatant from this centrifugation; pH 5 3

$$ {}^*C = \frac{K}{\text{mg. N per cc. test solution}} \cdot \quad K = \frac{1}{t}\log\frac{a}{a-x} \cdot $$

† Temperature 37°.

‡ Temperature 30°

TABLE III

Demonstration of Enzymatic Component, Pepsinase, in Renin Preparations

	N	K^*	C†	$\dfrac{C_{\text{CGluT}}}{C_{\text{CGluP}}}$
	mg			
Carbobenzoxy-l-glutamyl-l-tyrosine	0 666	0 00036	0 00054	2.16
Carbobenzoxy-l-glutamyl-l-phenylalanine	0 666	0 000165	0 00025	

The acidity was maintained at pH 5 3 by means of 0 1 N citrate buffer.   Incubation temperature 30°   The enzyme solution employed was the same as Preparation B, Table II

$$ {}^*K = \frac{1}{t}\log\frac{a}{a-x} $$

$$ \dagger C = \frac{K}{\text{mg. N per cc. test solution}} \cdot $$

reported in Table III.   The value of 2.16 for the proteolytic quotient (activity ratio) identifies its major component as swine kidney pepsinase. The reciprocal of this quotient was reported to be 0.50 by Fruton, Irving, and Bergmann (16).

Since prolonged dialysis did not seem to decrease the renin activity, and

since we were unable to determine pepsinase activity accurately in the starting material, our attention was focused on attempts to separate or correlate these two activities in the dialyzed sample. The fractionation procedure most frequently employed in the purification of renin seems to be its precipitation with acetone from aqueous or saline extracts of kidney. Examination of Table IV reveals a very large decrease in renin activity upon repeated precipitation with this solvent but also a corresponding decrease of pepsinase activity. The quotient C_{RS}/C_{CGluT} had changed but slightly, and the variation 18.5×10^4 to 19.4×10^4 is within the limits of the experimental error. When the acetone-precipitated material was allowed to stand in the ice box for several weeks, pepsinase activity decreased to less than one-half its former value, while the renin activity remained unchanged. The rise in the value of the proteolytic quotient from

TABLE IV

Effect of Acetone Precipitation on Renin Activity

	$C^* \times 10^4$ Carbobenzoxy-glutamyl-tyrosine (CGluT)†	C^* Renin substrate (RS)‡	$\dfrac{C_{RS}}{C_{CGluT}} \times 10^{-4}$
1st pptn with acetone (pseudoglobulin)	4.8	93	19.4
2nd " " " "	2 3	43	18.5
Same material after standing at 5° for 3 wks	1 12	43	38.0

$$* \; C = \frac{K}{\text{mg. N per cc. test solution}} \cdot \quad K = \frac{1}{t} \log \frac{a}{a-x} \cdot$$

† pH 5 3, incubation temperature 30°

‡ pH 6 5, incubation temperature 30°

18.5×10^4 to 38.0×10^4 is no longer within the experimental error of the method and indicated that a fractional inactivation of pepsinase had taken place.

In regard to methods for purification of renin, it becomes evident that repeated acetone precipitation is not desirable because of the large loss of renin activity, nor can enzymatic homogeneity be attained by this treatment. Trypsinase activity becomes entirely suppressed with and without cysteine activation, and aminopeptidase activity was not followed during these acetone precipitations but it would be safe to say that it will be retained with little or no loss, since leucylpeptidase at least can be purified by repeated precipitation with acetone (21).

A number of related phenomena may be pointed out. The main argument for the identity of renin and pepsinase activity appears to be that crystalline pepsin can be substituted for renin, suggesting that their mode

of action is similar; since pepsin and intracellular pepsinase are homospe-
cific, this should also be true for renin and pepsinase.   Against this concept
the following three objections may be raised.

1. Intracellular swine kidney pepsinase appears to be a heat-sensitive
enzyme which is rapidly inactivated at temperatures above 30°, while
renin can be exposed to 40° without appreciable loss of renin activity.

2. Prolonged standing at 5° causes considerable loss of pepsinase activ-
ity but renin activity remains unchanged.

3. Pepsin causes inactivation of angiotonin (22) probably by hydrolysis
of a peptide linkage sensitive to it.   This inactivation can be demonstrated
in two ways: (a) by incubation of angiotonin with pepsin and (b) by in-
cubating pepsin with renin substrate for a prolonged period of time, which
causes a significant decrease in the yield of pressor substance.   Intracell-
ular pepsinases which are homospecific when compared to pepsin must be
expected to exert the same action on angiotonin as well as to show a de-
crease in the yield of pressor substance when incubated with renin substrate
for excessive periods, yet it is well known that renin samples can be pre-
pared, which are entirely devoid of angiotonase activity.

The evidence presented above therefore indicates that renin and pep-
sinase activity must be attributed to different chemical entities, which,
although not identical, may be homospecific.   This possibility becomes
even more probable when it is remembered that pepsin, shown to be homo-
specific with pepsinase, can be substituted for renin in the preparation of
a pressor substance which is similar to angiotonin (4).   The validity of
this concept can be tested by comparing the action of renin and pepsinase
upon the same substrate and interpreting the data in the light of Berg-
mann's specificity concept.

If Bergmann's definition of the proteolytic coefficient (6) is correct, it
follows that the ratio of proteolytic coefficients for two homospecific en-
zymes when the same substrate is used must be a constant. Using the sym-
bols C_A^I and C_A^{II} for the coefficients of enzymes I and II acting upon sub-
strate A, we obtain the ratio

$$\frac{C_A^I}{C_A^{II}} = \frac{(p_E)_I(a \cdot b \cdot r)}{(p_E)_{II}(a \cdot b \cdot r)} = \frac{(p_E)_I}{(p_E)_{II}} = \text{constant} \tag{1}$$

Thus, for any single substrate, suitable for hydrolysis by the class of homo-
specific enzymes under investigation, the ratio of the proteolytic coefficients
must be same. Bergmann and his associates have demonstrated the
correctness of this extended concept.   The ratio of the C values for pan-
creatic carboxypeptidase and swine kidney carboxypeptidase when carbo-
benzoxyglycyl-l-phenylalanine (CGlyP) is used is

$$\frac{C_{CGlyP}^{PCP}}{C_{CGlyP}^{SKCP}} = \frac{6570}{34} = 193$$

and the same ratio for carbobenzoxyglycyl-*l*-tyrosine (CGlyT)

$$\frac{C_{CGlyT}^{PCP}}{C_{CGlyT}^{SKCP}} = \frac{3620}{19} = 190$$

The values agree within the experimental error of the method (5, 15).

If renin and pepsin and, by inference, renin and pepsinase are homospecific enzymes, the same conditions must prevail provided a suitable peptide is used as substrate. The two enzymes to be considered are renin and pepsin both acting on renin substrate and both hydrolyzing carbobenzoxy-*l*-glutamyl-*l*-tyrosine If the proteolytic coefficients for renin and pepsin on renin substrate are C_{RS}^{R} and C_{RS}^{P} and the coefficients for the same enzymes on carbobenzoxy-*l*-glutamyl-*l*-tyrosine are C_{CGluT}^{R} and C_{CGluT}^{P}, it follows from the theory that

$$\frac{C_{RS}^{R}}{C_{RS}^{P}} = \frac{C_{CGluT}^{R}}{C_{CGluT}^{P}} \tag{2}$$

If this condition is not met, the two enzymes under consideration are not homospecific and it must be concluded that they would occupy different places in the scheme of classification suggested by Bergmann and Fruton (5).

It must be pointed out that the numerical value of the proteolytic coefficient is of little use without specification of the pH at which the hydrolysis took place. The effectiveness, that is the proteolytic coefficient, of a certain enzyme preparation varies with the pH of the medium, usually exhibiting one or two maxima at pH values characteristic of both enzyme and substrate. For most intracellular proteinases, the pH maximum is around 5.0 or 5.4, with little or no variation when different synthetic substrates are used, but with pepsin the maximum coefficient varies widely with pH and substrate. The hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by pepsin has a maximum at pH 4.0, while carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is maximally hydrolyzed at pH 4 5 (20). A proteolytic quotient C_{CGluT}/C_{CGluP} of 0.5 is a characteristic of all pepsinases but appears to be true for pepsin at pH 4.0 only. Intracellular pepsinases and pepsin are, therefore, homospecific if the quotient for pepsin is determined at pH 4.0, which happens to be the optimum pH value for the hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by this enzyme.

To illustrate the change of this quotient at different pH values, we have followed the course of the hydrolysis of these two characteristic substrates under the influence of pepsin at pH 4.1 and 5.3. The data presented in

Table V illustrate the complete reversal of the quotient at pH values other than 4.1.

Realizing the significance of the factors involved in the specificity concept, we return to Equation 2. The question now arises, which value of the proteolytic coefficient should be substituted in this equation in order to yield significant results which can be interpreted as proving hetero- or homospecificity of renin and pepsin?

It must be evident from the above discussion, especially from the example of pepsinase and pepsin, that for correct application of Equation 2 the pH value at which the proteolytic coefficient should be determined must be the one at which the correct *quotient* is obtained. For renin samples (pepsinase activity) pH 5.3 was found to yield a satisfactory ratio of,
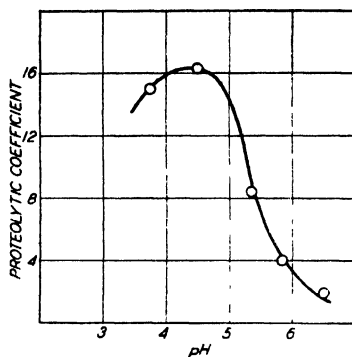
Fig. 1 Proteolytic coefficients (C_{RS}^{P}) for the formation of pepsitensin from renin substrate and crystalline pepsin plotted against the pH of the incubation mixture. The values were determined in the same manner as described for renin (7).

C_{CGluT}/C_{CGluP}, while, for the determination of C_{RS}^{R}, the optimum hydrolysis was reported as at pH 6.5 by Plentl, Page, and Davis (23). The proteolytic coefficient for carbobenzoxy-l-glutamyl-l-tyrosine and pepsin must be determined at pH 4.1 for reasons discussed above, but no theoretical deductions can be made as to the pH value at which C_{RS}^{P} should be determined. The determination of the proteolytic coefficients over the whole pH range seemed indicated and these are given in Fig. 1, where C_{RS} is plotted against the pH of the reaction mixture.

Since pepsin destroys angiotonin as well as pepsitensin, it must be assumed that the over-all picture of pepsitensin formation from renin substrate and pepsin consists of two consecutive reactions, in principle analogous to the mechanism of angiotonin formation when crude kidney extracts are used as enzyme. In an earlier paper (7) we analyzed this type

of reaction mechanism from a kinetic point of view and have shown how such an analysis can be utilized for the determination of both enzyme activities, despite the fact that they are in competition with each other. Pepsin incorporates both enzyme activities; *i.e.*, for each of the consecutive reactions, it acts on a different substrate.

$$\text{Renin substrate} \xrightarrow{k_1} \text{pressor substance} \xrightarrow{k_2} \text{vaso-inactive substance}$$

The two reaction constants, k_1 and k_2, divided by the pepsin concentration in mg. of N per cc. of test solution, will give the proteolytic coefficients C_{RS} and C_{PT} respectively. The determination of the two reaction constants is difficult and tedious, so that whenever possible a simplification of the method is applied. If k_2 is very small, k_1 can be determined by application of first order reaction kinetics, provided the incubation times are sufficiently short.

It soon became apparent that the yield of pressor substance did not decrease very much on prolonged incubation of the renin substrate-pepsin mixture, suggesting that the angiotonase or pepsitensinase activity is very small. C_{RS} was therefore determined according to the method for the determination of angiotonase-free renin (7).

Another condition for the validity of Equation 2 is the identity of the enzyme preparation employed for the determination of each of the coefficients. Since we have used crystalline pepsin, the coefficients C_{RS}^P and C_{CGluT}^P represent absolute values, which is not true for the analogous coefficients when renin is used as the enzyme. Having demonstrated that fractional denaturation of kidney extracts may result in loss of pepsinase activity without a corresponding loss of renin and knowing that there are a large number of intracellular enzymes present in such tissue extracts, we focus our attention on just three of them, two homospecific pepsinases and renin. The question to be decided is whether one of the pepsinases is identical with renin or whether there are really three distinct enzymes present. The fact that renin is not identical with one of the pepsinases does not necessarily exclude identity with the other. It seems that as the pepsinase activity decreases it reaches a certain value which cannot be lowered without corresponding loss of renin activity; *i.e.*, the quotient C_{RS}/C_{CGluT} does not change with further fractionation and the sample is therefore homogeneous with respect to these two activities. We can assume that a renin preparation which gives this characteristic quotient will be free of one of the pepsinases, and this is the sample that must be used for the determination of the coefficients in Equation 2. A renin preparation satisfying these requirements gave a proteolytic coefficient of 43 for C_{RS} and 1.12×10^{-4} for C_{CGluT}.

Examination of Fig. 1 reveals that C_{RS} has one maximum at about pH 4.5. Taking the C_{RS} at pH 4.5 as 16.0 and substituting this value in Equation 2 together with the value for C_{RS}^{R} derived above, we obtain

$$\frac{C_{RS}^{R}}{C_{RS}^{P}} = \frac{43}{16} = 2.7 \tag{3}$$

The theory demands that this value must be equal to the ratio of the proteolytic coefficients of carbobenzoxy-l-glutamyl-l-tyrosine when this is

TABLE V

Action of Crystalline Pepsin on Carbobenzoxy-l-glutamyl-l-tyrosine and Carbobenzoxy-l-glutamyl-l-phenylalanine at Different pH Values

Citrate buffers were used to maintain the pH at the desired value. Incubation temperature 30°.

	pH	Pepsin N per cc test solution	K^*	$C\dagger$	Average $\dfrac{C_{CGluT}}{C_{CGluP}}$
		mg.			
Carbobenzoxy-l-glutamyl-l-tyrosine	4.1	0.892	0.000213	0.000239	
		1.158	0 000488	0.000205	
Carbobenzoxy-l-glutamyl-l-phenylalanine	4.1	0.892	0 000097	0.000119	2 2
		0.892	0.000102	0.000115	
Carbobenzoxy-l-glutamyl-l-tyrosine	5.3	1.27	0.0000798	0.000063	
		1.27	0.0000710	0.000051	
Carbobenzoxy-l-glutamyl-l-phenylalanine	5.3	0.88	0.000203	0.00023	0.24
		1.28	0.000362	0.00028	

$* K = \dfrac{1}{t} \log \dfrac{a}{a-x}.$

$\dagger C = \dfrac{K}{\text{mg. N per cc. test solution}}.$

hydrolyzed by renin and pepsin. Using the data from Table V for pepsin and for renin, the values previously reported, we obtain

$$\frac{C_{CGluT}^{R}}{C_{CGluT}^{P}} = \frac{1.12 \times 10^{-4}}{2.25 \times 10^{-4}} = 0.5 \tag{4}$$

This value is not comparable with the one found for Equation 3 and the difference is obviously beyond the experimental error of the method. Since we have taken the maximum value for C_{RS}^{P}, it is evident that for different pH values of the reaction mixture Equation 3 would yield an even larger number for the ratio of the coefficients. This would make the suggested homospecificity of renin and pepsinase even less likely.

It should be pointed out that taking the maximum value of C_{RS}^P for the calculation of the quotient in Equation 3 represents the most favorable condition for the attempted demonstration of homospecificity. The only way in which this ratio could approach the value of Equation 4 would be by lowering the pH for the renin-renin substrate reaction. We are not justified in doing this. Even if it were done, within reasonable limits, the quotient for renin substrate would still be several times greater than the quotient for carbobenzoxy-l-glutamyl-l-tyrosine. Thus, under the most favorable circumstances, the possibility of homospecificity of renin and pepsinase is excluded. We are, therefore, forced to the conclusion that the enzyme, renin, is heterospecific when compared to pepsin or intracellular pepsinases.

SUMMARY

1. A number of typical synthetic substrates were incubated with renin preparations. The resulting hydrolyses of these substrates indicated the presence of carboxypeptidases, pepsinases, trypsinases, and aminopeptidases.

2. Prolonged dialysis of renin preparations against distilled water resulted in elimination of the carboxypeptidase component. Precipitation of the protein components of the dialyzed renin preparation with acetone failed to alter the proteolytic quotient (pepsinase to renin activity) and caused at least 50 per cent loss in renin activity with each precipitation. On prolonged standing of this material, the same proteolytic quotient increased 2-fold, indicating that renin activity of kidney extracts cannot be attributed to their pepsinase components.

3. The action of crystalline pepsin, known to be homospecific with pepsinases, and renin was compared, renin substrate and carbobenzoxy-l-glutamyl-l-tyrosine being used as substrates. Since the ratio of the proteolytic coefficients of renin and pepsin on renin substrate under the most favorable circumstances was found to be many times greater than the ratio of the analogous coefficients for carbobenzoxy-l-glutamyl-l-tyrosine, it was concluded that renin and swine kidney pepsinase are heterospecific enzymes.

BIBLIOGRAPHY

1. Braun-Menendez, E., Fasciolo, J. C., and Leloir, L. F., *Am. J. Med. Sc.*, **200,** 608 (1940).
2. Braun-Menendez, E , Fasciolo, J. C., Leloir, L. F., and Muñoz, J. M., *J. Physiol.*, **98, 283** (1940).
3. Croxatto, H., and Croxatto, R., *Science*, **95,** 102 (1942).
4. Croxatto, H., Croxatto, R., and Alliende, J., *Rev. Soc. argent. biol.*, **18,** 441 (1942).
5. Bergmann, M., and Fruton, J. S., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1,** 63–96 (1941).
6. Bergmann, M., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2,** 49–69 (1942).
7. Plentl, A. A., and Page, I. H., *J. Exp. Med.*, **78,** 367 (1943).
8. Northrop, J. H., Crystalline enzymes, New York (1939).
9. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145,** 253 (1942)
10. Bergmann, M., and Zervas, L., *Z. physiol. Chem* , **224,** 1 (1934)
11. Koenigs, E., and Mylo, B , *Ber. chem. Ges.*, **41,** 4427 (1909)
12. Bergmann, M , Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, **111,** 225 (1935).
13. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130,** 81 (1939).
14. Van Slyke, D. D., Dillon, R T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141,** 627 (1941)
15. Bergmann, M , and Fruton, J S., *J. Biol. Chem* , **145,** 247 (1942).
16. Fruton, J. S., Irving, G. W., and Bergmann, M., *J Biol. Chem.*, **141,** 763 (1941).
17. Rocha e Silva, M., and Andrade, S. O , *J. Biol. Chem* , **149,** 9 (1943).
18. Katz, Y. J., and Goldblatt, H., *J. Exp. Med.*, **78,** 67 (1943).
19. Hofmann, K., and Bergmann, M., *J. Biol. Chem* , **134,** 225 (1940).
20. Fruton, J. S., and Bergmann, M , *J. Biol Chem.*, **127,** 627 (1939).
21. Johnson, M. J , Johnson, G H , and Peterson, W H., *J Biol. Chem.*, **116,** 515 (1936)
22. Plentl, A A., and Page, I. H., *J. Exp. Med.*, **79,** 205 (1944).
23. Plentl, A. A , Page, I H., and Davis, W W., *J. Biol Chem.*, **147,** 143 (1943).

ON THE ENZYMATIC SPECIFICITY OF RENIN

II. THE ACTION OF CRYSTALLINE PEPSIN ON PEPSITENSIN AND ANGIOTONIN

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In Paper I of this series (1) we have shown that the enzymatic component of swine kidney responsible for pepsinase activity is not identical with the enzyme renin. This was demonstrated by fractional denaturation of pepsinase, leaving the renin activity of the sample practically unaltered. Since fractional denaturation does not necessarily exclude homospecificity of the two or more enzymes under investigation, we have eliminated this possibility by comparing the action of crystalline pepsin and renin on the same substrates; *viz.*, carbobenzoxy-*l*-glutamyl-*l*-tyrosine and renin substrate. The ratio of the proteolytic coefficients of renin and pepsin on renin substrate was found to be many times greater than the ratio of these coefficients when carbobenzoxy-*l*-glutamyl-*l*-tyrosine is used as substrate. For two homospecific enzymes this ratio is a characteristic constant, from which it follows that renin and kidney pepsinase are neither identical nor homospecific.

Two enzymes may exhibit widely different characteristics; *i.e.*, appear heterospecific in their behavior towards a number of synthetic substrates of known structure, and yet produce the same split-products when allowed to act on them. Renin and pepsinase or renin and pepsin, although heterospecific in their proteolytic behavior, may still give rise to the same physiologically active substance. Although pharmacological evidence presented by Alonso, Croxatto, and Croxatto (2) suggested identity of pepsitensin and angiotonin, convincing chemical evidence to that effect is lacking.

Since pepsitensin is inactivated by pepsin (2, 3), a quantitative study of the course of this reaction similar to our investigation of the action of pepsin and other crystalline proteolytic enzymes on angiotonin (4) might aid in deciding whether angiotonin and pepsitensin are or are not identical.

Methods and Materials

Crystalline Pepsin—This was prepared according to the procedure of Northrop (5). A stock solution of this enzyme was made by dissolving 4.0 gm. of the nearly dry material in 100 cc. of normal saline. The nitrogen

content of this solution was determined by micro-Kjeldahl analysis and usually was in the range of 5.00 to 5.50 mg. of N per cc.

Angiotonin—This material was prepared as previously described (6).

Renin Substrate—A fraction of hog serum (1.5 to 2.10 M ammonium sulfate) was used for this purpose. The experimental procedure for the preparation of this protein fraction was essentially the one described by Plentl, Page, and Davis (7). A concentrated solution (5 to 10 per cent) was thoroughly dialyzed against tap water, distilled water, and normal saline. It was then diluted to contain 2.0 per cent of total protein as determined by the biuret method of Kingsley (8).

Pepsitensin—A solution of renin substrate was adjusted to pH 5.8 with dilute sulfuric acid and incubated with crystalline pepsin for 10 minutes at 30°. 700 mg. of crystalline pepsin were used per liter of renin substrate. The pH of the solution usually dropped slightly during the incubation. After the required length of time, the temperature was rapidly raised to 95° and kept at this temperature for at least 15 minutes. The coagulated proteins were removed by filtration through coarse filter paper and the clear filtrate was concentrated under reduced pressure. This concentrated solution usually contained some denatured proteins which were removed by two or three filtrations through a Seitz pad. The final preparation was a pale yellow solution similar in appearance to the usual angiotonin samples. For reasons of convenience, the solutions were standardized to exhibit approximately the same pressor strength as our standard angiotonin. Such a standard preparation raised the arterial pressure of a pithed cat 50 to 60 mm. of Hg upon intravenous injection of 0.1 cc. of pepsitensin or angiotonin.

Incubation—The determination of the reaction constant was carried out as previously described for the determination of angiotonase (9). The pH of all solutions, *i.e.* angiotonin, pepsitensin, pepsin, and normal saline, was adjusted with hydrochloric acid or sodium hydroxide to the desired value before they were mixed. 1.0 cc. of the substrate (pepsitensin or angiotonin) was diluted to 4.0 cc. with normal saline and placed in a constant temperature bath at 30° for at least 20 minutes. 1 cc. of the pepsin solution, diluted to contain the desired amount of enzyme, was then added, and the solutions were thoroughly mixed and incubated for a definite length of time, usually ranging from 5 to 60 minutes. The reaction was stopped by adjusting the pH of the solution to 5.5 and immediately immersing the vessel in a beaker of boiling water. After removal of the coagulated proteins by filtration through coarse paper, the clear filtrate was assayed for its pressor strength by intravenous injection into a pithed cat. Comparison with a control (without enzyme) gave the percentage of angiotonin or pepsitensin destroyed.

First order reaction constants were calculated according to the equation

$$K = \frac{2.3}{t} \log_{10} \frac{100}{100 - \% \text{ destruction}}$$

and the proteolytic coefficient C was obtained by dividing the reaction constant by the concentration of the enzyme expressed in mg. of N per cc. of test solution. Proteolytic coefficients referring to angiotonin as substrate are designated as C_A, while those obtained with pepsitensin appear as C_{PT}.

TABLE I

Comparison of Action of Pepsin on Pepsitensin and Angiotonin at pH 4.5

No buffer was used but all solutions were adjusted to this pH before being mixed. The factor F is the ratio of blood pressure rise obtained with the sample to the blood pressure rise obtained with the control   Incubation temperature 30°.

Substrate	Incubation time	F	Pepsin N per cc test solution	K^*	$C\dagger$
	min		*mg.*		
Angiotonin	20	0.23	0.089	0.063	0.89
	60	0	0.089		
	25	0.29	0.053	0.048	0.91
	40	0.17	0.053	0.042	0.80
Average					0.87
Pepsitensin	30	0.70	0.178	0.0118	0.066
	60	0.53	0.178	0.0107	0.060
	20	0.68	0.356	0.0184	0.052
	30	0.60	0.356	0.0175	0.049
	45	0.43	0.356	0.0185	0.052
Average				.	0.056

$$* K = \frac{2.3}{t} \log_{10} \frac{100}{100 - \% \text{ destruction}}.$$

$$\dagger C = \frac{K}{\text{mg. pepsin N per cc. test solution}}.$$

The results of a comparison of pepsitensin and angiotonin under identical conditions appear in Table I. Both substrates were acted upon by the same pepsin sample at the same pH (4.50) and with the same test animal. In all other instances, the results of which are recorded graphically in Fig. 1, different test animals and various preparations of crystalline pepsin were used.

DISCUSSION

The question of identity or non-identity of these two substances deserves considerable attention because of a number of implications which have an

important bearing upon the structure of angiotonin. For example, the inactivation of angiotonin by chymotrypsin (4) can best be explained by assuming that the angiotonin molecule contains at least 1 aromatic amino acid residue in a terminal position, *i.e.* contributing one of the free amino groups, and that chymotrypsin acts as an exopeptidase (aminopeptidase) rather than an endopeptidase. If an enzyme with the characteristics of a pepsinase (pepsin or swine kidney pepsinase) can form angiotonin from a specific globulin, this globulin must be assumed to contain at least one peptide linkage composed of an acid and aromatic amino acid residue where that portion containing the aromatic amino acid radical constitutes the
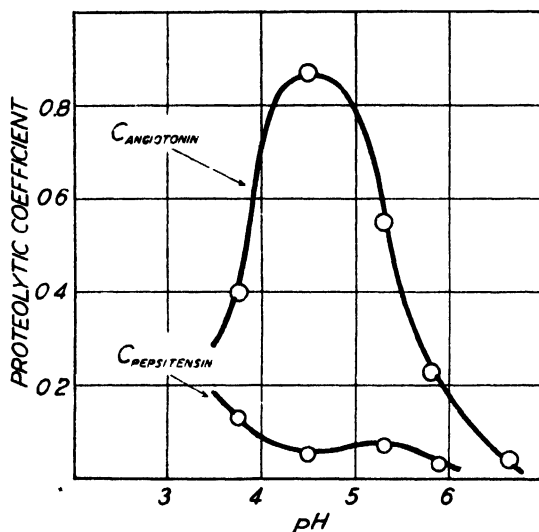
FIG. 1. Proteolytic coefficients for the reaction of crystalline pepsin on pepsitensin and angiotonin as a function of pH. The incubation temperature in all the experiments was 30°. The hydrogen ion concentration was maintained at the values indicated by the use of citrate buffers.

angiotonin molecule. Hydrolysis of this linkage results in the formation of angiotonin (and pepsitensin) and an inactive polypeptide. If the two pressor substances angiotonin and pepsitensin are identical, the hypothesis of a terminal aromatic amino acid radical finds one more piece of supporting evidence. If they prove to be different substances, the hypothesis of a terminal aromatic amino acid residue cannot be dismissed until further evidence to the contrary can be furnished.

Two heterospecific enzymes may split the same substrate on the same sensitive peptide bond, giving rise to the same products. It is conceivable that the action of renin and pepsinase (or pepsin) on renin substrate may

produce the same pressor substances, angiotonin and pepsitensin, and the demonstration of the heterospecificity of renin and pepsinase (1) is therefore of little help if it is to be decided whether or not angiotonin and pepsitensin are identical.

The two substances show no difference in their pharmacological behavior, as demonstrated by Alonso, Croxatto, and Croxatto (2). Their experiments indicate that qualitatively the pressor action of pepsitensin and angiotonin is the same and that both are destroyed by trypsin, tyrosinase, amino oxidase, "renal hypertensinase," and crystalline pepsin. Braun-Menendez, Fasciolo, Leloir, Muñoz, and Taquini stated in a recent article (10) that pepsitensin and angiotonin are closely related substances but are not identical. They based their conclusion on the observation that angiotonin is inactivated by "hypertensinase" prepared from red blood cells, while pepsitensin appears to be resistant to this enzyme.

We have carried out a number of preliminary experiments in an attempt to show some difference in their chemical behavior but all general color reactions usually associated with angiotonin preparations, e.g. Sakaguchi's or Pauly's reaction, are also given by pepsitensin with approximately the same intensity. Neither of these two reactions has so far been demonstrated to depend solely upon the angiotonin molecule because of the lack of a chemically pure substance. Most preparations contain free amino acids (5) and a positive Sakaguchi reaction may therefore be wholly or in part attributed to free arginine, while Pauly's reaction may be associated with histidine, imidazole-containing compounds, or aporrhegmas of histidine. It, therefore, became apparent that to settle the question of identity or non-identity of pepsitensin and angiotonin a careful quantitative study seems indicated.

Since both substances are destroyed by prolonged action of proteolytic enzymes upon them, we thought a comparison of the rate constants might give the desired information. The proteolytic coefficient is the product of two factors, one of which is characteristic of the enzyme and the other a function of the substrate. If, therefore, the same enzyme preparation is used on both substrates and if these substrates are identical, the proteolytic coefficient of the two reactions must be identical over the whole pH range. The experimental data in Table I and Fig. 1 demonstrate a distinct difference in the rate of inactivation of pepsitensin and angiotonin.

The effective pH range for both experiments was assumed to be from pH 3.0 to 6.5; these limits were determined by the stability of the substrate and the enzyme. Pepsin loses some of its activity above pH 6.0 (5), while angiotonin seems to be slowly inactivated below pH 3.0. The proteolytic coefficients for pH values above 6.0 are therefore only indicative and should not be relied upon for the reasons given.

Another characteristic feature of both substrate and enzyme is the pH

value at which the proteolytic coefficient is a maximum. Broadly speaking, the optimum pH for the hydrolysis of peptides by pepsin is somewhere between 2.0 and 5.0, whereas this value can be narrowed to a range of 4.0 to 4.5 for most peptides of known structure. In a previous publication (4), we reported this maximum for angiotonin to be about 5.5, which on more careful examination appears to be somewhat too high. Our present data for pepsitensin at various pH values indicate two maxima, one at about pH 5.5 and the other below 3.5. This finding does not represent an unusual phenomenon, for similar instances are recorded in the literature. The fact that pepsitensin exhibits two pH maxima cannot be assumed to have any bearing upon the substrate structure. This phenomenon and its interpretation are not well understood and, like the magnitude of the proteolytic coefficient, appear to be a characteristic of both enzyme and substrate (11). Hofmann and Bergmann (12) found benzoylargininamide to show one maximum when hydrolyzed by crystalline trypsin and Rocha e Silva and Andrade (13) showed that the same substrate had two maxima when acted upon by papain trypsinase.

Thus, pepsitensin and angiotonin differ, not only in their resistance to peptic hydrolysis in a quantitative way, but also show a qualitative difference in regard to the optimum acidity for such a hydrolysis.

Since both pressor substances are hydrolyzed or inactivated by crystalline pepsin, pepsitensin as well as angiotonin must contain the requisite number and arrangement of amino acids. The sensitivity of a peptide bond towards peptic hydrolysis seems to be dependent upon a free carboxyl; i.e., a dicarboxylic amino acid such as glutamic or aspartic acid in combination with an aromatic amino acid such as tyrosine or phenylalanine (14). Since pepsin is an endopeptidase and as such inactivates pepsitensin, the requisite dipeptide moiety must be located in the center of the molecule; i.e., at least the dicarboxylic amino acid must have its amino group involved in a peptide linkage. Although the speed of hydrolysis of pepsitensin is considerably lower than that of angiotonin by the same enzyme, it is, nevertheless, about 5000 times as fast as the hydrolysis of carbobenzoxy-l-glutamyl-l-tyrosine. The latter represents a typical pepsin substrate commonly employed for the measurement of peptic activity.

Since it was demonstrated that the two substances are not identical, a further implication follows which may be of interest. Since kidney pepsinase and pepsin are homospecific enzymes and since the product of peptic hydrolysis differs in its chemical constitution from angiotonin, it follows that the action of kidney or spleen pepsinase upon renin substrate should result in the formation of pepsitensin and not angiotonin. Crude kidney extract if employed as a source of renin will, therefore, produce a mixture of pepsitensin and angiotonin. Since the pepsinase content of such a mix-

· ture is usually very small, the amount of pepsitensin produced will be almost negligible when compared to that of angiotonin. To furnish experimental evidence for this hypothesis would be difficult at the present time, for kidney pepsinase is not known in chemically or enzymatically homogeneous form. Such preparations, if at all free of renin, contain unduly large amounts of aminopeptidase and trypsinases, both of which inactivate the product so efficiently that its presence cannot be demonstrated.

Regarding the nature of the difference between angiotonin and pepsitensin, no definite conclusion can be drawn at the present time. Both substances are probably polypeptides, differing in the number rather than in the nature of the amino acid residues of which they are composed.

SUMMARY

Since renin and kidney pepsinase are not homospecific enzymes, the identity of pepsitensin and angiotonin appears questionable. Pepsitensin and angiotonin were both found to be inactivated when incubated with varied amounts of crystalline pepsin, but showed a distinct difference in the magnitude of the proteolytic coefficients as well as the hydrogen ion optima for these reactions. Further, with pepsitensin as substrate two hydrogen ion optima were obtained, one at pH 5.5 and the other below pH 3.5, while only one such optimum could be demonstrated with angiotonin. It was concluded that, although angiotonin is very much more rapidly inactivated by pepsin than pepsitensin, both substances contain a similar arrangement of amino acids favorable for peptic hydrolysis. Pepsitensin and angiotonin are not identical and probably differ in the number, rather than in the nature, of amino acids residues of which they are composed.

BIBLIOGRAPHY

1 Plentl, A. A., and Page, I H., *J. Biol. Chem.*, **155**, 363 (1944).
2. Alonso, O., Croxatto, R , and Croxatto, H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 61 (1943).
3. Croxatto, H., and Croxatto, R., *Rev. Soc. argent. biol.*, **17**, 439 (1941).
4. Plentl, A. A., and Page, I. H., *J. Exp. Med.*, **79**, 205 (1944).
5. Northrop, J. H., Crystalline enzymes, New York (1939).
6. Page, I. H., and Helmer, O. M., *J. Exp. Med.*, **71**, 29 (1940).
7. Plentl, A. A., Page, I. H., and Davis, W. W., *J. Biol. Chem.*, **147**, 143 (1943).

8. Kingsley, G. R., *J. Lab. and Clin. Med.*, **27**, 6, 840 (1942).
9. Plentl, A. A., and Page, I. H., *J. Exp. Med.*, **78**, 367 (1943).
10. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Muñoz, J. M., and Taquini, A. C., *Rev. Soc. argent. biol.*, **19**, 304 (1943).
11. Bergmann, M., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 49 (1942).
12. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 81 (1939).
13. Rocha e Silva, M , and Andrade, S. O., *J. Biol. Chem.*, **149**, 9 (1943).
14. Fruton, J. S., and Bergmann, M., *J. Biol. Chem* , **127**, 627 (1939).

THE THROMBOPLASTIC ACTIVITY OF TISSUE PHOSPHATIDES*

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It has long been known that tissue cells contain an agent which by converting prothrombin to thrombin initiates the process of blood coagulation. This factor, or better, these factors, known by a multiplicity of names, have been described by their several rediscoverers as either (a) thermolabile and soluble in water (1–4), or (b) thermostable and soluble in alcohol and ether (5). The innumerable controversies between the adherents of these two groups were finally resolved by the recognition that both were right.

For a discussion of the water-soluble factor, the thromboplastic protein, reference may be made to previous publications from this laboratory (6, 7). The agent soluble in organic solvents was recognized to be associated with the phosphatides (8–11) and, more particularly, with the cephalin fraction (10, 12) of animal tissues. Plants, and even microorganisms, appear to contain similar substances (13). Later investigations of the thromboplastic lipids (14–16) confirmed their association with the crude cephalin fraction, but the significant finding was made (14) that the active agent could be extracted from the cephalin preparations by means of alcohol. During an investigation of the phosphatides isolated from blood platelets it was furthermore observed that the fractionation of the active cephalin fraction by means of alcohol resulted in the accumulation of most of the activity in the alcoholic mother liquors (13). Essentially similar results were obtained in a study of the phosphatides contained in preparations of the thromboplastic protein from beef lungs (17).

All these findings served to throw considerable doubt on the conception that the thromboplastic lipid was identical with the phosphatide traditionally termed cephalin, *viz.* ethanolamine phosphoryl diglyceride; and, with the discovery of the complex nature of brain cephalin (18, 19) and of other tissue phosphatides (20), the question of the identity of the active lipid once more became open.

The purpose of the study presented here was twofold. One of its objectives was to ascertain whether any of the better characterized components of tissue cephalin were responsible for the thromboplastic effect.

This point may be considered settled with respect to phosphatidyl serine which, in its purified state, was found completely devoid of thromboplastic activity. As regards ethanolamine phosphoryl diglyceride, for which, in the opinion of this author, the term cephalin ought to be reserved, it is much more difficult to arrive at a definite statement. All that can be said at present is that purified preparations from beef brain containing a large proportion of this phosphatide exhibit a thromboplastic activity corresponding to $+++$ (compare the explanation in Table I). But in view of the elusive nature of the thromboplastic lipids and of the much higher potency of some of the lipid fractions isolated from heart muscle, which will be discussed later, it is felt that the activity of beef brain cephalin may well be due to impurities.

The second task consisted in the exploration of methods that, with the guidance of activity measurements, would lead to the concentration of the active fraction. It involved an expedition into the underbrush of phosphatide chemistry, which was far from inviting, since it was soon found that, the activity usually went with the least pure phosphatide fractions which showed the greatest deviation from the analytical expectations. It is not unlikely that the clotting activator (its lipid nature can by no means yet be affirmed) was present in a small concentration in even the most potent lipid preparations.

The experiments here described were carried out with phosphatide preparations isolated from pig heart, which yielded the most potent material, from beef heart, and beef brain. An inspection of the characteristics of the thromboplastically most active lipid samples encountered (Fractions 5, 8, and 9 in Table I; Fraction 4 in Table III; Fractions 7 and 8 in Table IV) shows them to have one property in common; namely, a remarkable solubility in absolute alcohol. It was necessary to freeze the alcoholic solutions, in some cases to $-60°$, in order to precipitate the active fractions. The analytical composition of the numerous lipid fractions examined failed to afford a clue to the nature of the thromboplastic lipids. Fractions 8 and 9 from pig heart (Table I), the most potent preparations encountered in this work, had an anomalously high nitrogen content, but this was not true of other, although less active, fractions. It proved equally unavailing to use the amino nitrogen contents or the iodine values as a guide.

In view of the solubility properties of the active lipids, it is curious that the thromboplastic activity usually is found in the lipid portion initially insoluble in alcohol, and not in the lecithin fraction which, when sufficiently purified, has been repeatedly shown to be inactive (compare (15)). This peculiar association cannot be explained at present.

It is hardly necessary to point to the difficulties which confront the

investigation of the chemical nature of the thromboplastic lipids. At the time when criteria of solubility and a satisfactory analytical composition were deemed sufficient for the identification of a phosphatide, the extremely optimistic identification of the thromboplastic lipid with what then was called cephalin was justified. But the studies presented in this paper show the situation to be considerably more complicated. It will, in a continuation of this work, be necessary to extend the examination not only to lipids known to be present in heart preparations, such as the acetal phosphatides (21) and cardiolipin (22), but also to other possible contaminants of which creatine (23, 24) may be mentioned as one example. It must, moreover, be borne in mind that any substance that is susceptible of phosphorylation in the organism may occur, if only in traces, in the form of a phosphatide.

Discussions of the complex chemical composition of tissue phosphatides, especially with regard to heart muscle, will be found in previous publications (20, 22, 24–28). At this point, attention may be drawn to a study, included in this article, of the recoveries of phosphorus and nitrogen in the hydrolysis of pig heart phosphatides, a discussion of which will be found in the experimental part.

The addition of alcohol to the concentrated petroleum ether extract of heart muscle produced the precipitation of a fraction that was almost free of nitrogen and phosphorus. This observation, already mentioned in a previous publication (20), was repeatedly made in all experiments with pig and beef heart discussed here. One of these fractions from pig heart was purified and examined in greater detail; it appears to be a triglyceride, probably oleodistearin.

In conclusion, attention should be directed to the entirely different level of activity of the thromboplastic protein, as compared with the active lipids (7). The difference is about 1000-fold: 0.03 γ of the thromboplastic protein shows the same clotting activity as 30 γ of the most potent lipid specimens. This makes the identity of the two activation mechanisms appear doubtful. (Compare the earlier discussion (6, 7).)

EXPERIMENTAL

Phosphatides from Pig Heart[1]

The minced tissue, weighing 2745 gm., was twice extracted with 2 liter portions of acetone. The organ powder then was treated with 2 liters of petroleum ether (b.p. 30–60°) for 5 days and this procedure repeated with

[1] The organs were in all cases obtained from freshly slaughtered animals and worked up without delay with the customary precautions (inert gas atmosphere, freshly rectified solvents, storage in the cold, etc). For the analytical procedures used, compare (20).

fresh solvent, followed by an extraction of the residue with 2200 cc. of absolute alcohol for 21 days. The dried residue from the extraction weighed 523 gm.

Petroleum Ether Extract (Table I)—The combined extracts, concentrated to 250 cc. and chilled, yielded the insoluble *protagon fraction* weighing 10.5 gm. The addition of 5 volumes of acetone to the concentrated petroleum ether supernatants produced a precipitate which was washed with ice-cold acetone and dried *in vacuo*. This material was flocculated from its suspension in 500 cc. of water by the addition of 750 cc. of acetone, centrifuged in the cold, washed with acetone, and dried. *Fraction 1*, 35.4 gm., was an almost white sticky wax. The treatment of a solution of the substance in 100 cc. of petroleum ether with 400 cc. of absolute alcohol resulted in the precipitation of *Fraction 2*, 27.3 gm. of a slightly yellowish powder of low N and P content. This fraction will be discussed later in this section.

The filtrate from Fraction 2 was evaporated *in vacuo* and 5 volumes of acetone were added to the solution of the residue in 35 cc of ether. The resulting precipitate, *Fraction 3*, weighed 4.7 gm. and formed a light brown hard paste. It may be mentioned that the iodine value of this substance dropped within 4 weeks from 82.4 to 51.9 and was after 3 more weeks found at 41.0 (compare (20)).

Fraction 3 was subjected to a fractionation from alcohol. The clear solution of 4.49 gm. of this material in 60 cc. of warm absolute alcohol deposited 1.37 gm. of *Fraction 4* at 0°. The alcoholic supernatant was chilled to −60° and rapidly centrifuged in a refrigerated angle centrifuge, when 0.81 gm. of *Fraction 5* was obtained. The mother liquor was evaporated, and the residue dissolved in petroleum ether and precipitated with acetone. This gave 1.74 gm of *Fraction 6*. These substances formed brittle brown glasses.

Alcohol Extract (Table I)—The extract was concentrated *in vacuo* and the residue dissolved in petroleum ether. The solution was dried, chilled, cleared, and concentrated. The addition of 4 volumes of absolute alcohol precipitated *Fraction 7*, 1.9 gm. of a yellowish wax. The clear alcoholic solution of the residue obtained by the evaporation of the filtrate from Fraction 7 deposited *Fraction 8* at 0°, weighing 0.28 gm. The chilling of the supernatant to −60° yielded 2.18 gm. of *Fraction 9*. The thick oil remaining from the evaporation of the mother liquor was treated with acetone at −60°, and the precipitate centrifuged and washed with acetone at −60°, when *Fraction 10* weighing 5.6 gm. was obtained. A portion of the acetone supernatant from Fraction 10 yielded on evaporation *Fraction 11*. All compounds formed light brown hard waxes.

The analytical data are summarized in Table I.

Glyceride Fraction (Fraction 2 in Table I)—The recrystallization of Fraction 2 from hot absolute alcohol resulted in the deposition of white microcrystalline needles in a yield of 91 per cent. This material was free of N, P, and S, melted at 41°, and had the following composition: C 74.6, H 11.7, glycerol[2] (as isopropyl iodide) 9.7, iodine value 26.1. (Required for oleodistearin (888.8), C 76.9, H 12.2, glycerol 10.4, iodine value 28.6, m.p. 42°.)

The saponification of 8.24 gm. of this material with boiling 5 per cent alcoholic potassium hydroxide resulted in the isolation of 80 mg. of unsaponifiable matter and 7.61 gm. of fatty acids which were separated in the

TABLE I

Pig Heart Phosphatides

Fraction No	P	Total N	N.P	NH₂-N	Iodine value	Thromboplastic activity*
	per cent	*per cent*		*per cent*		
1	0.70	1.06		0.44	40.9	+
2	0.23	0.31			37.1	−
3	3 79	1.73	1.0	0.84	82.4	++
4	3.64	1 50	0.9	0.73	56.3	+
5	3.56	1 66	1 0	0.56	82 0	+++
6	4.03	1 88	1 0	0 51	53.1	+
7	3 37	2 38	1.4	1.33	40.5	−
8	3.06	3.59	2.5	1.52	73.5	++++
9	3 51	2 52	1 6	1.05	73.6	++++
10	3.38	2.72	1.8	1.14	71.4	−
11	2 83	2 28	1 8	0 48	79.8	−

* The coagulation tests were carried out at 30 5° with 0 1 cc. of rooster plasma and 0.03 cc. of serial dilutions of the lipids in m/15 phosphate buffer of pH 6 8. − no thromboplastic activity. + clot with 120 γ of lipid in more than 40 minutes; ++ within 30 to 40 minutes, +++ within 20 to 30 minutes ++++ clot with 30 γ of lipid within 10 to 20 minutes The normal clotting times of the plasma specimens employed were invariably above 150 minutes.

usual manner (lead salts in alcohol) into 5.73 gm. of solid and 1.17 gm. of liquid fatty acids. The solid acids, recrystallized twice from methyl alcohol, once from ethyl alcohol, and twice from acetone, yielded 2.35 gm. of a crystalline product melting at 67°, solidification point 66°. The mixture of this compound with pure *stearic acid* (m.p. 69°) melted at 68°. The equivalent weight of this compound (0.05 N alcoholic KOH standardized with pure palmitic acid, naphtholphthalein as indicator) was 281.6 (required for C₁₈H₃₆O₂, 284.5).

Nitrogen and Phosphorus Balance (Table II)—It appeared of interest

[2] We are indebted to Mr. W. Saschek for these analyses.

to follow the recovery of nitrogen and phosphorus from the hydrolysis of a representative sample of heart phosphatides. For this purpose, a sample of mixed phosphatides was used, whose preparation from pig heart has been described previously (Preparation 5 in Table I (20)). This material had the following composition: P 3.70, N 1.58, NH_2-N 0.83, amino acid N 0.11, iodine value 86.0. At the time of the hydrolysis the NH_2-N value of the 3 months old sample had dropped to 0.38 per cent (compare (20)).

For hydrolysis 11.6600 gm. of the preparation were refluxed with 250 cc. of 2 N H_2SO_4 for 48 hours. The aqueous hydrolysate was freed of the fatty acids by repeated extraction with ether, the ether layer was several times washed with 2 N H_2SO_4 and water, and the washings were combined with the main solution which was adjusted to a volume of 500 cc. (Table

TABLE II

Phosphorus and Nitrogen Recoveries in Hydrolysis of Pig Heart Phosphatides

Fraction No.	Fraction	P		Total N		NH₂-N	
		Weight	Per cent of total	Weight	Per cent of total	Weight	Per cent of total
		mg		*mg*		*mg*	
	Total, subjected to hydrolysis (11 6600 gm)	431 5	100	184.3	100	96 8	100
1	Aqueous hydrolysate (500 cc.)	407.0	94 3	152.0	82 5	58.8	60.7
2	Fatty acids (8 3524 gm)	15.7	3 6	31 9	17 3	30 0	31 0
3	Barium salts (0 8271 gm)	26 2	6 1	0 8	0.4	0	0
4	Alcohol-insoluble (0 0778 gm.)	0	0	0 6	0.3	0	0
5	Alcoholic solution of base chlorides (100 cc)	9.1	2.1	109 0	59.1	44.0	45.5

II, Fraction 1). The analytical data for the fatty acid fraction, 8.3524 gm. of a brown semisolid oil, will be found as Fraction 2 in Table II.

The aqueous hydrolysate (486 cc.) was concentrated to one-half its volume *in vacuo* and adjusted to pH 10 by the addition of finely powdered $Ba(OH)_2$; the mixture was neutralized by means of CO_2 and freed of the precipitate. The addition of 2 volumes of absolute alcohol to the filtrate precipitated the barium salts, weighing 827.1 mg. (Table II, Fraction 3). The filtrate from this fraction was acidified with HCl and taken to complete dryness *in vacuo;* the residue was dissolved in absolute alcohol, 77.8 mg. of insoluble material (Table II, Fraction 4) were removed, and the alcoholic solution of the base chlorides adjusted to a volume of 100 cc. (Table II, Fraction 5).

The analytical figures, summarized in Table II, are of interest in various

respects. They confirm the previously reported finding (20) that the drop in amino nitrogen, of phosphatides during storage was due to a masking of the free amino groups rather than to their destruction. The values for the total phosphatide sample subjected to hydrolysis, given in Table II, are based on the analytical results obtained for the fresh preparation. 3 months later, when the balance experiment was carried out, the values for P and total N had remained practically unchanged, but the amino nitrogen value had, as pointed out, dropped to 0.38 per cent. If the calculations were based on the latter figure, the amino nitrogen recovered after hydrolysis in Fractions 1 and 2 (Table II) would correspond to twice as much as was contained in the sample before hydrolysis.

It is furthermore noteworthy that the large amount of nitrogen retained in the fatty acid fraction (Table II, Fraction 2) was practically all in the form of amino nitrogen (compare (26, 29, 30)). That the nitrogen content of this fraction was not due to unhydrolyzed phosphatide is indicated by its low phosphorus value. The nature of the phosphorus compound present in the alcoholic solution of base chlorides (Fraction 5) is unknown.

Phosphatides from Beef Heart

Two freshly obtained beef hearts were cleaned and ground. The minced tissue, weighing 3523 gm., was twice extracted with acetone and then treated with two 3 liter portions of petroleum ether for a period of 3 and 7 days respectively. The dried extraction residue weighed 559 gm.

The concentration and chilling of the combined petroleum ether extracts led to the deposition of the *protagon fraction*, 60.8 gm. of a white crystalline material. The filtrate was concentrated to 300 cc. and 4 volumes of absolute alcohol were added, when *Fraction 1* (Table III) separated, 174.4 gm. of a half liquid paste of low P and N content. The supernatant was concentrated to 85 cc. and 2 volumes of acetone containing 300 mg. of magnesium acetate were added. The heavy oil which separated was dried, emulsified in 150 cc. of physiological saline, and precipitated by the addition of 0.5 volume of acetone. The precipitate was repeatedly washed with cold acetone-saline (1:2) and acetone and carefully dried at a low temperature *in vacuo* over P_2O_5; *Fraction 2* (Table III), 24.4 gm. of a very soft salmon-colored paste, was obtained.

The major part of Fraction 2 (22.5 gm.) was subjected to a fractionation from alcohol, as has been described in a preceding section for the pig heart preparation. *Fraction 3* (3.95 gm.) separated at 0°, *Fraction 4* (7.41 gm.) at −60°. Both substances were reddish brown, soft pastes. The residue obtained by the evaporation of the supernatant from Fraction 4 was treated

with acetone. *Fraction 5* separated as a light yellow soft paste, weighing
9.19 gm., at 0°; the acetone mother liquor yielded *Fraction 6*, 1.63 gm. of a
yellow oil. Fractions 4 and 6 gave a strong blue color when treated with
ninhydrin (in the presence of a trace of pyridine); Fraction 5 showed only
weak ninhydrin reaction. Only Fraction 4 exhibited flocculation with
salmine at pH 7 (31).

An attempt at the further purification of *Fraction 4* involved the treat-
ment of 6.92 gm. of this material with 50 cc. of warm methyl alcohol. The
portion insoluble at 0° was reprecipitated with methyl alcohol from its
solution in petroleum ether, yielding *Fraction 41* (Table III), weight 1.65
gm. This material did not form a sufficiently stable suspension in saline
to permit its assay for thromboplastic activity. The combined mother
liquors were evaporated and the residue was dissolved in 75 cc. of methyl

TABLE III

Beef Heart Phosphatides

Fraction No	P	Total N	N:P	NH₂-N	Iodine value	Thromboplastic activity*
	per cent	*per cent*		*per cent*		
1	0.34	0 14				
2	3 21	1.40	1.0	0 77†	90.0	
3	3.12	1 03	0 7	1 18	102.0	+
4	3.01	1 24	0.9	0 88	86.0	+++
41	2.43	1 40	1.3	1.40	87.3	
42	3.51	1.44	0 9	1 71	105.0	+++
43	2.97	1 67	1 2	0 63	84.0	−
5	3 71	1 60	1 0	0 47	94.3	−
6	2 39	0 97	0 9	0 75	91 3	−

* See Table I for an explanation of the procedure
† This material contained no amino acid N

alcohol. The addition of 1.5 cc. of a saturated aqueous sodium chloride
solution to the centrifuged methyl alcoholic solution produced a precipitate
which was centrifuged off, washed with cold methyl alcohol (containing
2 per cent of saturated NaCl solution), and precipitated from its clear solu-
tion in ether by means of acetone. The yield was 0.47 gm. of *Fraction 42*.
The supernatant yielded *Fraction 43*, 3.05 gm. of a yellow wax. The at-
tempted fractionation of the cadmium chloride double salt of this material
by distribution between petroleum ether and 80 per cent alcohol (22) was
unsuccessful, practically all being soluble in the latter solvent.

The analytical data will be found summarized in Table III. It may be
pointed out that two fractions which had particularly high iodine values
(Table III, Fractions 3 and 42; and also Fraction 2 in Table IV) gave
anomalous amino nitrogen figures (compare (32)).

Phosphatides from Beef Brain

The tissue mince, weighing 2490 gm., was twice treated with 3 liter portions of acetone at 4°. This was followed by an extraction with 3 liters of absolute alcohol for 24 hours at 4°, and with two 3 liter portions of petroleum ether (b.p. 30–60°) for 48 hours each at room temperature, in the dark. The extraction residue weighed 271 gm. In another experiment, 8320 gm. of minced beef brain were similarly treated with acetone and alcohol. The alcoholic extracts obtained in both experiments were united and worked up jointly.

Alcohol Extract (*Table IV*)—The extract (about 15 liters) was chilled in ice and freed of a small amount of cerebrosides by filtration through a large fritted glass immersion filter. The precipitate, produced by the addition to the filtrate of 150 gm. of cadmium chloride in 100 cc. of water, was separated from the chilled mixture, washed with ice-cold alcohol, and suspended in 1000 cc. of petroleum ether. A large part of the lecithin double salt was removed by twenty-six extractions with 200 cc. portions of 80 per cent alcohol (22). The clear petroleum ether layer was concentrated to a volume of 140 cc. and 10 volumes of absolute alcohol were added. The thick sirup which separated from the cooled mixture was freed from the supernatant by decantation and again precipitated in the same manner. The precipitate, collected in a refrigerated centrifuge, was dissolved in 300 cc. of petroleum ether and again subjected to twenty-seven extractions with 75 cc. portions of 80 per cent alcohol. The petroleum ether solution, which at this stage appeared almost free of the lecithin-cadmium chloride complex, was evaporated to dryness *in vacuo*. The precipitate obtained by the addition of 500 cc. of absolute alcohol to the clear solution of the evaporation residue in 50 cc. of petroleum ether was sedimented by centrifugation in the cold, dissolved in 50 cc. of benzene, and again precipitated with 200 cc. of ethyl acetate. After one more precipitation from a benzene solution with 4 volumes of an alcohol-ethyl acetate mixture (1:1), the material was dissolved in chloroform and freed of Cd by the addition of 18 per cent ammonia in methyl alcohol in the usual manner. The concentrated supernatant was again treated with ammonia, centrifuged, and evaporated to dryness *in vacuo*. The residue was immediately taken up in petroleum ether and the solution, after having been cleared by centrifugation, washed five times with a 10 per cent NaCl solution in water, briefly dried with anhydrous sodium sulfate, and evaporated *in vacuo*. The addition of 60 cc. of absolute alcohol to the petroleum ether solution of the residue (6 cc.) produced a copious precipitate which was removed by the centrifugation of the chilled mixture and subjected to several precipitations with absolute alcohol from its clear solution in ether and in chloroform. This material, 1.22 gm. of an almost white powder, is entered as *Fraction 1* in Table IV.

The united alcoholic mother liquors were evaporated *in vacuo*, the residue dissolved in ether, and acetone was added to the clear concentrated ethereal solution. The resulting precipitate, *Fraction 2*, 0.20 gm. of a yellowish soft paste (Table IV), differed from lecithin in the difficulty with which it could be suspended in water.

Petroleum Ether Extract (Table IV)—The precipitate obtained with 5 volumes of alcohol from the extract, following its concentration and liberation of cerebrosides in the usual manner, was suspended in physiological saline and flocculated by the addition of 0.5 volume of acetone. It then was precipitated from an aqueous suspension by means of 1 N hydrochloric acid. This was followed by precipitation from an ethereal solution with 5 volumes of acetone. The flocculation of the phosphatides was, under

TABLE IV

Beef Brain Phosphatides

Fraction No.	P	Total N	N:P	NH₂-N	Amino acid N	Iodine value	Thromboplastic activity*
	per cent	*per cent*		*per cent*	*per cent*		
1	3.69	2.09	1.3	1 50		88 3	—
2	4.01	1.92	1.1	2.11		111	—
3	3.85	1 76	1.0	1.57	0.69	83 0	—
4	3.97	1.65	0.9	1.51	0.83	67.1	++
5	3.88	1.65	0.9	1.64	0.79	69.6	—
6	3.81	1.80	1.0	1.67	0.71	78.0	++
7	3.81	1.91	1.1	1.59	0.51	76.0	+++
71	3.45	1.74	1.1	1.40		57.0	—
72	3.61	1.90	1.2	1.50		72.5	+++
8	4.04	2.06	1.1	1 46	0.33	78 7	+++
81	2.46	1.68	1.5	0.54		59 1	—
82	4.03	1 97	1.1	1 26		73.9	+++

* See Table I for an explanation of the procedure.

these conditions, found to proceed much less readily, probably because of the preceding removal of the cations with acid. Sedimentation of the precipitate in the refrigerated angle centrifuge yielded *Fraction 3*, 20.2 gm. of an almost white hard wax (Table IV).

The addition of 150 cc. of absolute alcohol to the solution of 19.5 gm. of this fraction in 100 cc. of chloroform brought about the separation of a yellow thick oil which, when treated with cold chloroform-alcohol (2:3), alcohol, and acetone, solidified to a yellowish powder, *Fraction 4*, weighing 6.20 gm.

The combined mother liquors were adjusted to an alcohol concentration of 75 per cent (by volume), when *Fraction 5* separated, 3.96 gm. of a light brown powder.

The evaporation residue of the filtrate from Fraction 5 yielded, on addition of 5 volumes of absolute alcohol to its solution in 20 cc. of chloroform, *Fraction 6*, 4.85 gm. of a soft light brown wax.

The mother liquors were evaporated *in vacuo* and the residue dissolved in 50 cc. of warm absolute alcohol. The solution, when cooled overnight, deposited a light yellow oil which on trituration with cold alcohol and acetone solidified to give 1.99 gm. of a yellowish sticky powder, designated *Fraction 7*. The oil resulting from the evaporation *in vacuo* of the alcohol filtrate was treated with cold acetone, when *Fraction 8* was obtained, 1.79 gm. of a yellow wax which soon discolored to brown. It may be noted that the division of Fraction 3 into five separate fractions involved a loss of only 3.7 per cent of the starting material.

The major portions of Fraction 7 (1.35 gm.) and Fraction 8 (1.23 gm.) were subjected to further fractionation. Each substance was dissolved in 30 cc. of warm methyl alcohol. The clear solutions deposited on cooling *Fraction 71* (0.54 gm.) and *Fraction 81* (0.22 gm.) respectively. To each of the supernatants 160 mg. of potassium acetate dissolved in 2 cc. of alcohol were added and the chilled mixture cleared by centrifugation and evaporated *in vacuo*. To the ethereal solutions of the residues 4 volumes of acetone were added, when *Fraction 72* (0.74 gm.) and *Fraction 82* (0.89 gm.) respectively separated. These substances which showed considerable thromboplastic activity were subdivided into a number of fractions, but the inactive, ill defined products obtained will not be described here.

The analytical properties of the lipid fractions isolated from beef brain are summarized in Table IV.

Phosphatidyl Serine from Beef Brain[3]

This substance was isolated from beef brain following, with the exception of certain details, the method of Folch (19). The best preparation obtained had the following composition: P 3.71, N 1.72, amino N 1.71, amino acid N 1.57, iodine value 63.4. The iodine values were invariably found higher than the figure of 39.8 reported by Folch.

All samples of phosphatidyl serine examined, including one kindly furnished by Dr. J. Folch of The Rockefeller Institute for Medical Research, were devoid of thromboplastic activity towards rooster plasma.[4]

Estimation of Thromboplastic Activity

In all experiments freshly obtained rooster plasma was employed by the technique customary in this laboratory (13). The plasma samples were

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[3] The preparation of this material was carried out by Mr. D. B. Sprinson.

[4] Dr. J. Folch had the kindness to supply a specimen of brain cephalin free of amino acids. This fraction, which according to a private communication from Dr. Folch contained total N 1.8, amino N 1.68, amino acid N 0.06, had a thromboplastic activity corresponding to +++ (compare the explanation in Table I).

extremely stable; without the addition of activating substances their clotting times never fell below 150 minutes. Their coagulability was tested by the addition of the thromboplastic protein from beef lungs (7), in order to exclude specimens of low prothrombin content. The lipid samples were directly suspended in M/15 phosphate buffer of pH 6.8 and tested in serial dilutions at 30.5°. Each tube contained 0.1 cc. of plasma and 0.03 cc. of the lipid suspension.

While it proved easy to demonstrate the presence or lack of thromboplastic activity in a given lipid sample and to distinguish between highly potent and slightly active fractions, the definition of an activity unit lending itself readily to comparison was impossible. In order to avoid the cumbersome presentation of protocols of individual assays, the schematic formulation adopted in Tables I, III, and IV was chosen. This procedure was found to give sufficiently well reproducible results.

SUMMARY

Numerous phosphatide fractions obtained by a variety of methods from pig heart, beef heart, and beef brain were examined for thromboplastic activity. The most potent preparations, especially those from pig heart, were very soluble in alcohol. Phosphatidyl serine from beef brain was inactive; brain cephalin itself (*i.e.* the ethanolamine-containing phosphatide fraction) showed some activity. The theoretical discussion of the findings presented here emphasizes the complex composition of tissue phosphatides and leads to the conclusion that the thromboplastically active lipids cannot yet be identified with any of the known phosphatides.

The crude lipid preparations from heart muscle were shown to contain considerable amounts of fat. One of these accompanying compounds, isolated from pig heart, was identified as a triglyceride, probably oleodistearin. A study of the distribution of phosphorus and nitrogen in the hydrolysis products of pig heart phosphatides and of the recoveries of these elements is likewise included.

BIBLIOGRAPHY

1. Wooldridge, L. C , Arch. Anat. u. Physiol., Physiol. Abt., 397 (1886).
2. Halliburton, W. D , Proc. Roy. Soc. London, 44, 255 (1888).
3. Conradi, H., Beitr. chem. Physiol. u. Path , 1, 136 (1902).
4. Fuld, E , Beitr. chem. Physiol. u. Path., 2, 514 (1902).
5. Schmidt, A., Zur Blutlehre, Leipzig, 99, 105 (1892); Weitere Beitrage zur Blutlehre, Wiesbaden, 231 (1895).
6. Cohen, S. S., and Chargaff, E , J. Biol. Chem , 136, 243 (1940).

7. Chargaff, E., Moore, D. H., and Bendich, A., *J. Biol. Chem.*, **145**, 593 (1942).
8. Wooldridge, L. C., *Arch. Anat. u. Physiol.*, *Physiol. Abt.*, **389** (1883).
9. Zak, E., *Arch. exp. Path. u. Pharmakol.*, **70**, 27 (1912).
10. Howell, W. H., *Am. J. Physiol.*, **31**, 1 (1912–13).
11. Bordet, J., and Delange, L., *Ann. Inst. Pasteur*, **27**, 341 (1913).
12. McLean, J , *Am. J. Physiol.*, **41**, 250 (1916); **43**, 586 (1917).
13. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *J. Biol. Chem.*, **116**, 237 (1936)
14. Zunz, E , and La Barre, J., *Arch. internat. physiol.*, **18**, 116 (1921); *Compt. rend. Soc. biol.*, **85**, 1107 (1921).
15. Gratia, A., and Levene, P. A., *J. Biol. Chem*, **50**, 455 (1922)
16. Fischer, A., and Hecht, E., *Biochem. Z.*, **269**, 115 (1934).
17. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **139**, 741 (1941).
18. Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).
19. Folch, J., *J Biol. Chem.*, **146**, 35 (1942).
20. Chargaff, E., Ziff, M., and Rittenberg, D , *J. Biol. Chem.*, **144**, 343 (1942).
21. Feulgen, R., and Bersin, T., *Z. physiol. Chem.*, **260**, 217 (1939).
22. Pangborn, M. C., *J. Biol. Chem* , **143**, 247 (1942).
23. Klenk, E., *Z. physiol. Chem* , **221**, 67 (1933).
24. Urban, F F , *Biochem Z* , **293**, 264 (1937)
25 Erlandsen, A., *Z physiol. Chem* , **51**, 71 (1907)
26 MacArthur, C G , Norbury, F G , and Karr, W. G , *J. Am. Chem. Soc.*, **39**, 768 (1917).
27 MacLean, H., and Griffiths, W. J , *Biochem J* , **14**, 615 (1920).
28. Thierfelder, H , and Klenk, E., Die Chemie der Cerebroside und Phosphatide, Berlin (1930)
29 MacArthur, C G , *J Am Chem. Soc* , **36**, 2397 (1914)
30. Koganei, R , *J Biochem* , Japan, **3**, 15 (1924)
31. Chargaff, E , *J Biol Chem* , **125**, 661 (1938)
32 Folch, J., Schneider, H A , and Van Slyke, D D , *Proc Am. Soc. Biol. Chem.*, *J Biol Chem* , **133**, p xxxiii (1940).

A METHOD FOR THE DETERMINATION OF SUBSTANCES ENZYMATICALLY CONVERTIBLE TO THE FACTOR STIMULATING STREPTOCOCCUS LACTIS R*

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Wright and Welch (1) reported that the incubation of fresh rat liver with xanthopterin, or with any one of a number of crude materials, resulted in an apparent production of folic acid. Totter, Mims, and Day (2) confirmed the finding that xanthopterin is able to promote the formation of folic acid by fresh rat liver *in vitro*. Furthermore, they found that incubation with fresh rat liver was capable of producing a 15-fold or greater increase in the folic acid content of a sample of dried brewers' yeast. Such data indicate that their sample of brewers' yeast contained a substance (or substances) which was not utilizable for growth by *Streptococcus lactis* R, but which became utilizable after incubation with fresh rat liver.

It was suggested (2) that in considering the nutrition of higher forms of life assays of "potential" folic acid would be more valuable than assays of preformed folic acid. However, in measuring the substances convertible into the factor stimulating *Streptococcus lactis* R by the use of fresh liver brei, certain difficulties are encountered. Chief among these is the fact that the amount of preformed *S. lactis* R-stimulating substances in rat liver is highly variable. Consequently, attempts have been made to prepare from rat liver a relatively stable preparation which could be used in the place of fresh rat liver. Such a liver preparation has been successfully made, and evidence has been accumulated which indicates that the active substance of the preparation has enzymic properties. The purpose of this paper is to describe the preparation of the rat liver extract and its use in the assay of substances convertible to the *S. lactis* R-stimulating factor.

Following the suggestions contained in a recent review (3) the authors have avoided the use of a specific name for this factor, since it appears that there are several related compounds with similar activity.

EXPERIMENTAL

Preparation of Liver Enzyme Extract

Stock Enzyme Solution—Add 30 to 40 gm. of fresh rat liver to 160 cc. of 0.05 M phosphate buffer, pH 7, and mix in a Waring blendor for 4 minutes.

Centrifuge for 20 minutes at high speed to remove any cellular parts.

Add ammonium sulfate to 50 per cent saturation (37.7 gm. per 100 cc.) and filter in the refrigerator.   Discard the precipitate.

Increase the ammonium sulfate to 80 per cent saturation (an additional 18.7 gm. per 100 cc. of filtrate), filter, and discard the filtrate.

Dissolve the precipitate in a small amount of phosphate buffer, pH 7, so that each cc. is equivalent to 0.7 to 0.9 gm. of original liver.

Free the extract from *Streptococcus lactis* R-stimulating substances by dialyzing in a cellophane membrane against the phosphate buffer at room temperature 8 hours with eight changes of buffer, and overnight in the refrigerator.

Add ammonium sulfate until 50 per cent saturation is again reached and store in the refrigerator under benzene.

This has been kept as long as 4 weeks without loss of activity.

Working Enzyme Solution—A 5 cc. quantity of the stock solution is again dialyzed against the phosphate buffer for 2 hours at room temperature with at least four changes.   Its activity is then tested by adding 0.025, 0.05, 0.1, and 0.2 cc. to tubes containing 100 γ each of Difco yeast extract[1] and then treating the tubes as described under "Procedure."   An amount that will give maximum values with a minimum blank is used for routine determinations.   (This should be 0.025 to 0.1 cc. if the preparation has been properly carried out.)   This solution also is stored under benzene in the refrigerator. It should be prepared twice a week.

Procedure

Tissue extracts are prepared as advised by Cheldelin *et al.* (4).   Various amounts of the extract or material to be assayed must be selected by estimate and by preliminary determination to give values of 10 to 20 millimicrograms of *Streptococcus lactis* R-stimulating substance.   Dilutions of the tissue extract should be made so that the amounts selected will be contained in 1 cc. of phosphate buffer, pH 7 (at least three levels should be chosen).

Set up Folin-Wu sugar tubes containing 1 cc. of tissue extract each and control tubes containing 1 cc. of the phosphate buffer solution only.

Add the required amount of working enzyme solution to each tube.

Incubate 4 hours at 37°.   The total volume should be the same in each tube and should not exceed 1.2 cc.

Precipitate the heat-coagulable proteins by placing the tubes in a boiling water bath for 3 minutes.   Gentle shaking aids flocculation of the proteins.

[1] Difco Bacto-yeast extract, Difco Laboratories, Detroit, Michigan.   Different samples of this preparation have varied in potency   The amount added should contain about 20 millimicrograms of material of potency 40,000 after incubation with the enzyme

Dilute to 25 cc. and mix.   Centrifuge.

The clear supernatant is assayed according to the method of Mitchell and Snell (5) for folic acid.   The data reported here were obtained by the use of a model 11 Coleman spectrophotometer.   Turbidities were measured after 18 hours incubation at 32°.

TABLE I

Assays of Various Materials for Streptococcus lactis R-Stimulating Factor, before and after Incubation with Rat Liver Enzyme

Material assayed	No of assays	S lactis R-stimulating factor	
		Preformed* (before incubation)	Potential* (after incubation)
		γ per gm	γ per gm.
Liver extract, Lilly, Sample A†	4	42	63
" " " " B	4	25–25.6	42–46
Yeast " Difco,‡ " A	(2) § 5	0.8–1.0	81.9–96
" " " ‡ " B	(4) § 20	2.5–3.5	193–214 (Average, 204)
Cabbage, Chinese	1	2.0	4.9
"	2	0.2, 0.9	1.4, 2.0
Carrot, root . .	1	0.42	0.84
" leaves	1	3.15	5.8
Chard	1	3.8	4.2
Bacto-peptone, Difco‡	2	0.63, 0.74	4.2, 5.4
Potatoes	1	1.03	1.5
Wheat, whole .	4	0.5–1.3	1.0–1.7

* Material of potency 40,000.

† This sample of liver extract, Lilly, has been used as a reference standard and was compared with a folic acid standard of potency 1, kindly furnished by Dr. H. K. Mitchell, University of Texas.

‡ Difco Laboratories, Detroit, Michigan.

§ The numeral in parentheses indicates the number of assays of preformed factor.

Assay Data

Typical assay data obtained with the method outlined above are recorded in Table I.   Each assay represents duplicate determinations at two or three levels.   The term "preformed factor" is applied to the amount of the growth factor as measured by direct assay.   The term "potential factor" is applied to the growth factor as measured after incubation with the rat liver enzyme.

It may be seen that material of both vegetable and animal origin contains amounts of potential factor greater than the corresponding amounts of preformed factor.   In almost every substance assayed there was found to be a significant increase in the factor after incubation with the rat liver

preparation. This increase varied from about 1.5-fold for potatoes to 100-fold for some samples of yeast extract.

DISCUSSION

It is evident from the data obtained that there is no constant relationship between the amount of preformed *Streptococcus lactis* R-stimulating factor and the amount which can be produced by the action of the enzyme preparation. Hence, values determined by direct assay according to published procedures may frequently be of little value when applied to the study of nutrition of higher animals, which presumably can bring about the conversion or can use the "potential factor" without alteration. It is not impossible that some species of microorganisms are also able to make use of the "potential factor."

It should be emphasized that the data in Table I have been obtained by the use of tissue extracts prepared with the aid of taka-diastase. The yeast extract was a cell-free, entirely water-soluble preparation. The action of the rat liver enzyme has been applied only to such extracts and not to the intact tissues. The action of the rat liver enzyme preparation is therefore not analogous to the action of taka-diastase or other enzymes which aid in the liberation of vitamins from tissues.

The purified rat liver enzyme preparation differs from liver brei in that the former is incapable of bringing about the "production" of *Streptococcus lactis* R-stimulating factor from xanthopterin.

It should likewise be pointed out that the method outlined above is subject to the disadvantage that, like all chemical and microbiological vitamin methods, the reliability of the data as a measure of the vitamin in the original tissue will depend upon the completeness of the extraction from the tissue.

SUMMARY

A relatively stable preparation of an enzyme, which is capable of producing the *Streptococcus lactis* R-stimulating factor from inactive material, has been made from rat liver.

A method of measuring the bacterial growth factor is described which involves the use of this rat liver enzyme preparation.

The term "preformed factor" is applied to the data as obtained by direct assay. The term "potential factor" is applied to data obtained in assays after incubation of the tissue extract with the rat liver enzyme.

Data obtained by the assay of extracts of several biological materials for preformed and potential *Streptococcus lactis* R-stimulating factor are reported.

BIBLIOGRAPHY

1. Wright, L. D., and Welch, A. D., *Science*, **98,** 179 (1943).
2. Totter, J. R., Mims, V., and Day, P. L., *Science*, in press.
3. Anonymous, *Nutr Rev* , **2,** 131 (1944).
4. Cheldelin, V. H., Eppright, M A , Snell, E. E , and Guirard, B. M., *Univ. Texas Pub., No. 4257*, 15 (1942)
5. Mitchell, H. K., and Snell, E. E , *Univ. Texas Pub., No. 4157*, 36 (1941).

COMPONENT FATTY ACIDS FROM THE FAT OF COW COLOSTRUM*

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In connection with a recent study on human milk fat (1) an opportunity was afforded to measure qualitatively and quantitatively the fatty acid distribution in the fat from human milk collected during the first 3 days of lactation. Some differences in composition were observed for these colostrum samples when they were compared with mature human milk fat. It was considered desirable therefore to make analyses of fat from cow colostrum in order to have available comparable data.

The results from the work with human milk fat indicated that as the period of lactation progressed from the 1st day's colostrum to mature milk the amount of phospholipid and high molecular weight acids decreased and the low molecular weight acids increased. In the present investigation of fat from cow colostrum no striking differences from published analyses for fat of mature cow's milk (2, 3) have been found. In fact the fatty acid analyses of the two are remarkably similar.

EXPERIMENTAL

Collection and Preparation of Fat for Analysis—The colostrum from which the fat was extracted for the present study was obtained from a single cow during the first 4 days after parturition. The cow had consumed a diet consisting of (a) about 12 pounds daily of mixed timothy and clover hay, (b) 30 pounds of corn silage, and (c) 10 pounds of a grain mixture for 2 months before the sample was collected. Variation in composition would be anticipated if the diet of the breed were altered. The milk was dried by the cryochem process and the lipids were extracted with alcohol and ether in a Soxhlet apparatus.

The phospholipids, removed from the total lipid material by the method of Bloor (4), amounted to 1.12 per cent of the total fat which was extracted. Phosphorus analysis (5) of the acetone-insoluble material indicated a phosphorus content of 3.96 per cent.

Analysis of Steam-Volatile Acids—The acetone-soluble lipids were saponi-

fied with potassium hydroxide in redistilled methanol. After removal of the solvent *in vacuo*, the dry soaps were dissolved in distilled water and transferred to a steam distillation apparatus. The soaps were then acidified with 50 per cent sulfuric acid and steam-distilled until 1500 ml. of aqueous distillate had been collected in an ice-cooled receiver.

The aqueous distillate was extracted with peroxide-free ethyl ether several times to remove the volatile fatty acids. After the ether extract had been dried with neutral anhydrous sodium sulfate, the ether was distilled through a fractionating column and the fatty acids were fractionally distilled at atmospheric pressure. A series of fractions was obtained which were analyzed for iodine values and neutral equivalents. Descriptions of the fractions are given in Table I. The aqueous portion of the steam dis-

TABLE I

Analytical Data for Fractions of Volatile Acids

Fraction No.	Weight	Iodine value (Wijs)	Neutral equivalent
	gm.		
V-1	2.316		*
V-2	0.276		*
V-3	0.1423		†
V-4	0.3225		165.5†
V-5	0.4223		90.8
V-6	0.2954		113.6
V-7	0.2600		112.8
V-8	0.3232		121.9
V-9	0.1850	0.1	135.0
V-R	0.4573	12.8	183.1

* Non-acidic material (ether).
† Mostly ether; acidity calculated as butyric acid.

tillate was titrated for total acidity, which was then calculated as weight of butyric acid. Also the sodium sulfate used in drying and the ether which was removed through the column were titrated with standard potassium hydroxide to determine the fatty acids as weight of butyric acid remaining in them. The butyric acid calculated from these three sources was 0.428 and 0.009 gm., respectively.

Analysis of Non-Volatile Acids—The acids non-volatile in steam were recovered and converted to neutral methyl esters which were then distilled at reduced pressure through a packed and electrically heated fractionating column. Analyses of the various methyl ester fractions were made for iodine values and saponification equivalents by standard methods and for percentages of octadecadienoic, octadecatrienoic, and eicosatetraenoic acids

by spectrophotometric examination of the soaps isomerized at high temperature. In Table II are shown the analytical results of the distillation. Absorption curves of the isomerized soaps (6) from several of the fractions are shown in Fig. 1. The typical absorption peaks at 234 mμ for conjugated diene material were present in all the fractions which contained

TABLE II

Analytical Data for Fractions of Methyl Esters from Non-Volatile Acids

Fraction No.	Weight	Iodine value (W₁Js)	Saponification equivalent	Methyl octadecadi- enoate	Methyl octadecatri- enoate	Methyl eicosatetra- enoate	n_{40}^{D}
	gm.			*per cent*	*per cent*	*per cent*	
1	0.209	4.7	182.8				1.4151
2	0.317	8.0	193.1				1.4204
3	0.533	6.8	203.7				1.4217
4	0.873	5.7	218.3				1.4251
5	1.140	7.3	232.0				1.4281
6	1.667	7.0	241.4				1.4289
7	1.635	10.2	243.7				1.4292
8	1.779	9.6	254.2				1.4324
9	1.305	13.2	267.9				1.4341
10	2.210	12.1	270.6				1.4336
11	2.880	7.7	271.0				1.4336
12	2.523	6.2	270.8				1.4336
13	6.244	3.9	270.8				1.4327
14	3.402	45.8	287.1	4.53	0.58		1.4388
15	2.057	58.8	294.8	7.63	1.14		1.4431
16	2.179	81.9	296.0	7.61	0.98		1.4436
17	1.942	81.3	296.4	7.04	0.85		1.4437
18	2.343	79.1	296.6	6.65	0.88		1.4436
19	2.045	76.5	296.8	5.75	0.72		1.4435
20	1.953	71.9	297.0	5.14	0.81		1.4433
21	1.842	66.0	297.3	4.65	0.77		1.4430
22	1.333	59.0	297.2	3.94	0.63		1.4416
23	1.840	50.6	297.0	3.36	0.59		1.4409
24	1.789	43.4	297.6	3.05	0.48		1.4408
25	1.039	106.2	305.7			13.9	1.4526
26	0.326	120.8	315.9			20.8	1.4587
Residue	0.802	95.9	475.1*				

* Neutral equivalent of free fatty acids, 329.2.

C₁₈ esters; the amounts of methyl octadecadienoate in Fractions 14 to 24 inclusive were calculated from these absorption maxima by ·comparison, after correction for triene absorption, with standard values obtained from the isomerization of methyl linoleate. The low peaks at 270 mμ, representing triene conjugation, were calculated as methyl octadecatrienoate by com-

parison of the absorption coefficients at 270 mμ with standard values for isomerized methyl linoleate. These values must be considered as indicating the maximum amount of triene absorption possible, for there was also some general absorption in this region, which would tend to make the apparent methyl octadecatrienoate greater than it actually was. It is entirely possible that the conjugated triene material which showed absorption at 270 mμ may have come from some source other than methyl octadecatrienoate, because a very small amount of triene absorption has been shown to arise during the handling and distillation of debromination linoleic acid (7). However, it is doubtful whether as much triene absorption as was found could possibly have arisen from the relatively small amount of methyl octadecadienoate present. Bosworth and Sisson (8) were unable to detect any
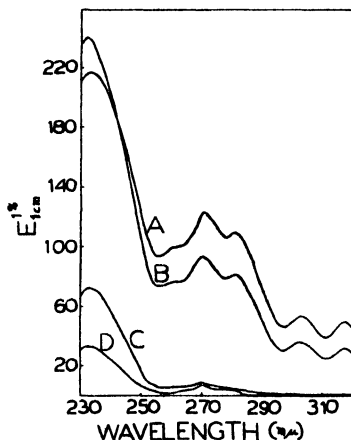
FIG. 1. Spectral absorption curves of the soaps resulting from the isomerization of several methyl ester fractions: Curve A, Fraction 26; Curve B, Fraction 25; Curve C, Fraction 16; Curve D, Fraction 24.

linoleic or linolenic acid in cow's milk fat by bromination studies, although Eckstein (9) does report the presence of small amounts of linolenic acid in various butter fats.

The absorption peaks at 304 and 317 mμ in the last fractions are characteristic of tetraene conjugation which was calculated as having been induced from methyl eicosatetraenoate with standard absorption values obtained for alkali-isomerized methyl arachidonate.[1] Inasmuch as the diene absorption in these latter fractions is much greater than can be accounted for by the eicosatetraenoic acid and the amount of C_{18} unsaturated mate-

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[1] Standard absorption values for isomerized methyl arachidonate were obtained from Dr. B. W. Beadle and Dr H. R. Kraybill, American Meat Institute.

rial is very small, there must have been some C_{20} and C_{22} dienoic acids present. For lack of a better method of determination the remainder of the C_{20} and C_{22} unsaturated material in these higher fractions was calculated "as C_{20} dienoic acid." All of the saturated acids in these higher fractions were calculated "as eicosanoic acid."

The compositions of the fractions which contained no esters of molecular weight higher than C_{18} were calculated directly from the data presented in Table II, and a summary of the component fatty acids in the entire colostrum fat is given as both weight percentage and molar percentage in Table

TABLE III

Component Fatty Acids of Fat from Cow Colostrum (and Mature Milk)

Acids	Colostrum fat		Butter fat (Hilditch and Longenecker (3))
	weight per cent	*molar per cent*	*molar per cent*
Butyric	2.6	7.2	8.1
Caproic	1.6	3.4	2.8
Caprylic	0.5	0.8	2.5
Capric	1.6	2.3	3.7
Lauric	3.2	3.9	4.4
Myristic	9.5	10.1	12.5
Palmitic	31 7	29.9	23.2
Stearic	11.8	10.0	7.6
"As eicosanoic"	0.6	0.5	1.0
Decenoic	0.1	0.2	0.4
Dodecenoic	0.2	0.2	0.5
Tetradecenoic	0.7	0 7	1.7
Hexadecenoic	2.7	2.5	3.7
Octadecenoic	28.5	24 3	24.8
Octadecadienoic	2.5	2 2	2 9
Octadecatrienoic	0.4	0.3	
Eicosatetraenoic	0.7	0.6	0.2
"As C_{20} dienoic"	1.1	0 9	

III. The fatty acid composition of the colostrum fat is very similar to that reported for typical butter fat (3) and the significant trend found in the component acids of human milk and colostrum fat (1) was not evident from this work.

SUMMARY

A sample of colostrum fat from a cow fed a known diet was analyzed for fatty acid composition. The component acids were found to be not significantly different from those of the fat of mature milk.

BIBLIOGRAPHY

1. Baldwin, A. R., and Longenecker, H. E., *J. Biol. Chem.*, **154**, 255 (1944).
2. Hilditch, T. P., and Paul, H., *Biochem. J.*, **30**, 1905 (1936).
3. Hilditch, T. P., and Longenecker, H. E., *J. Biol. Chem.*, **122**, 497 (1937–38).
4. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
5. Fontaine, T. D., *Ind. and Eng. Chem.*, *Anal. Ed.*, **14**, 77 (1942).
6. Mitchell, J. H., Jr., Kraybill, H. R., and Zscheile, F. P., *Ind. and Eng. Chem.*, *Anal. Ed.*, **15**, 1 (1943).
7. Wood, T. R., Jackson, F. L., Baldwin, A. R., and Longenecker, H. E., *J. Am. Chem. Soc.*, **66**, 287 (1944).
8. Bosworth, A. W., and Sisson, E. W., *J. Biol. Chem.*, **107**, 489 (1934).
9. Eckstein, H. C., *J. Biol. Chem.*, **103**, 135 (1933).

A COMPARISON OF THE RELATIONSHIP OF LACTIC ACID AND PYRUVIC ACID IN THE NORMAL AND DIABETIC DOG*

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The Embden-Meyerhof scheme (1) is widely accepted at the present time as a main avenue of carbohydrate breakdown for most tissues of the body. In this system pyruvic acid is a necessary intermediary both for the formation of lactic acid and for the oxidation of carbohydrate. Various observers have, however, reported that lactic acid can be produced in the diabetic animal from glucose independently of the formation of pyruvic acid (2, 3). Recent work of Bueding and coworkers (4) has shown that pyruvic acid does not accumulate in the blood of the diabetic animal after the administration of glucose until insulin has been injected, after which it appears in large quantities. This indicates that insulin acts at some intermediary stage between glucose and pyruvic acid. Klein (5) has observed that in the resting diabetic patient the levels of pyruvic acid and lactic acid rise and fall together. In the experiments of Klein, however, the diabetic patients were not entirely deprived of their insulin apparatus and, since they had been receiving protamine zinc and plain insulin until 14 to 24 hours before the experiment, it was not possible to conclude whether or not this relationship between the two metabolites would still hold in the complete absence of insulin. The use of completely depancreatized animals in the present investigation presented the writers with the opportunity to perform a crucial experiment on theoretically adequate preparations to determine whether lactic acid accumulates in the resting animal independently of pyruvic acid and in the absence of insulin, or whether the changes in the levels of both metabolites are correlated.

Method

Two sets of experiments were performed on unanesthetized dogs: one on sixteen normal trained dogs and the other on dogs depancreatized under

aseptic precautions. The animals were restrained on animal boards during the experiments to permit as little movement as possible.

Normal Dogs—After a control sample of blood has been drawn, 2 gm. per kilo of glucose were injected intravenously and further samples were drawn 15, 30, 60, 90, and 120 minutes after the injection. A detailed description of the diet and care of these dogs is presented in another paper (6).

Depancreatized Dogs—These dogs were subjected to the same procedure as the normal animals, except that after the collection of the 90 minute sample insulin was injected and further collections were made 15, 30, 60, and 120 minutes after this injection. Three of the dogs were depancreatized and used 48 to 96 hours postoperatively without ever having received injections of insulin. In order to avoid effects due to postoperative disturbances present in these depancreatized animals, four other dogs were depancreatized and maintained in good health by injecting regular insulin twice daily after their meals and by including pancreatin in their diet. These animals received no food or insulin 18 to 42 hours preceding the experiment.

Chemical Analysis—2 cc. of blood were drawn into a tuberculin syringe from the femoral artery. 1 cc. of this was expressed directly into chilled 10 per cent trichloroacetic acid to be analyzed for pyruvic acid by the method of Friedemann and Haugen (7). The 2nd cc. was freed from protein by Somogyi's technique (8) and after centrifugation the supernatant fluid was analyzed for glucose by the Hagedorn and Jensen method (9) and for lactic acid according to Barker and Summerson (10).

Results

The averages of the values for the sixteen normal dogs for the blood levels of lactic acid, pyruvic acid, and glucose following the injection of glucose are presented in Table I. Following the rapid rise in glucose resulting from the intravenous injection, there is a fast fall towards the control value during the 1st hour and then a slower return to the control during the next hour. The level of glucose is much higher than indicated immediately after the injection, for by 15 minutes, the interval after which the first sample is drawn, the value is already on its way down. The lactic acid and pyruvic acid curves show a delay between their swings and that of the glucose curve. They both reach their maximum at 30 minutes and then fall to approximately their control levels over the 2 hour period. The lactic acid and pyruvic acid blood levels follow the same general pattern and the ratio between them exhibits only comparatively small variations.

For comparison with these curves on normal animals we have the results

of experiments obtained in depancreatized dogs. In Table II are presented the curves for lactic acid, pyruvic acid, and glucose in dogs studied from 48 to 96 hours after pancreatectomy without having ever received supportive injections of insulin. Table III discloses the concentrations of the same substances in dogs that have been maintained for several weeks on insulin. These dogs were used 18 to 42 hours after the last dose of

TABLE I

Glucose Tolerance Tests (in Mg Per Cent) on Sixteen Normal Dogs

Normal animals	Control	15 min	30 min	60 min	90 min	120 min
Glucose	83	462	264	123	95	93
Lactic acid	6 8	10 0	10 7	7.9	7 0	6 2
Pyruvic acid	0 9	1 2	1 3	1 1	1 1	0 9
Ratio, lactic-pyruvic acid	7 9	8 2	8 0	6 9	6.5	6.8

TABLE II

Values of Glucose, Lactic Acid, and Pyruvic Acid Blood Levels for Dogs Depancreatized 48 to 96 Hours; No Insulin Injected

The results are expressed in mg per cent

Dog No		Glucose, 2 gm per kilo					Insulin				
		Control	15 min	30 min	60 min	90 min	15 min	30 min	60 min	90 min	120 min
1	Glucose	279	615	500	420	300	330	297	273	276	309
	Lactic acid	8 8	10 9	8.3	12 3	10 7	15.6	16 2	27 2	43 5	47 0
	Pyruvic acid	2 3	2.1	1 9	1.9	2 1	2 6	2 7	3 7	4.5	5 4
	Lactic-pyruvic	3 0	5 2	4 4	6.5	5 1	6 0	6.0	7.4	9 7	8 7
2	Glucose	252	670	520	455	415	315	237	174	180	183
	Lactic acid	8.4	8 1	7.2	4 9	5.6	3 3	8.6	15.3	17.6	27 9
	Pyruvic acid	1 7	2 1	1.7	1 7	1 7	1 8	1.7	2 1	2 8	3.3
	Lactic-pyruvic	5 0	3 9	4.2	2 9	3 3	1 8	5.0	7.3	6.3	8 4
3	Glucose	321	790	625	545	495	447	453	438	435	501
	Lactic acid	13 6	13 6	13.6	14.4	15 1	16 0	24.7	28 4	34 0	49.1
	Pyruvic acid	2.3	2.3	2.4	2.5	2.6	3.5	3 5	3 5	3 0	3.6
	Lactic-pyruvic	5.9	5.9	5 6	5 7	5.8	4.5	7 0	8 0	11.3	13.3

insulin. Table II shows that the dogs studied within a few days after the operation responded to the intravenous injection of glucose by changes in the concentration of lactic acid and pyruvic acid that were small and inconsistent in comparison with the enormous increases observed after the injection of insulin. These animals exhibited a relationship between the concentration of lactic acid and pyruvic acid which in 93 per cent of the

determinations did not differ significantly from the ratios of the normal animals. The observations presented in Table III, made on dogs maintained in good health by the injection of insulin, led, with certain exceptions, to the same results as the observations made on the animals used immediately postoperatively. They were similar in so far as the changes in lactic acid and pyruvic acid were small and inconsistent before an injection of insulin in comparison with the large increases which occurred following its injection. Both in the dogs receiving no insulin and in those maintained with insulin following pancreatectomy, insulin caused the

TABLE III

Values of Glucose, Lactic Acid, and Pyruvic Acid for Depancreatized Dogs Maintained on Insulin But Not Injected for 18 to 42 Hours before These Observations

The results are expressed in mg per cent.

Dog No		Glucose, 2 gm per kilo					Insulin				
		Control	15 min	30 min	60 min	90 min	15 min	30 min	60 min	90 min	120 min
4	Glucose	327	705	640	530	370	318	213	111	93	72
	Lactic acid	9 5	9 8	10 7	6 5	8 1	11 9	23 5	16 5	18.1	20.5
	Pyruvic acid	1 7	1 5	1.5	1 2	1 2	2 5	2 6	1 8	2 0	1 7
	Lactic-pyruvic	5 5	6.6	7.0	5 4	6 7	4 8	9 0	9 2	9 1	12 1
5	Glucose	303	725	690	655	590	489	366	292	141	87
	Lactic acid	7 7	11 6	21 4	13 5	17 2	21 9	27 0	25 1	20 0	13 0
	Pyruvic acid	1 1	1 1	1 3	1 0	1 2	2 5	2 3	2 3	2 3	1 8
	Lactic-pyruvic	7 3	10 6	17 0	13 5	14 2	8 7	11 9	10 9	8.8	7 3
6	Glucose	303	720	630	495		405	300	192	114	84
	Lactic acid	20.2	21 4	22.3	13 0		23 7	32 4	43 7	27 6	22 3
	Pyruvic acid	2 6	2 0	2.1	1 2		3 4	4.0	4 8	3.4	2 8
	Lactic-pyruvic	8 0	10.8	10 9	10 9		7.1	8 0	9 1	8.2	7 9
7	Glucose	462	1030	885	695	680	339	585	393	258	180
	Lactic acid	10.7	12.6	13.5	11.9	6 5	10 7	21.2	44.6	33 2	31 2
	Pyruvic acid	1 4	1.4	1.1	1 0	1 1	1 4	2.1	6.6	1 9	2.0
	Lactic-pyruvic	6.3	9.3	11 6	9 9	10.5	6.9	9 6	9 7	8 7	9.1

concentrations of lactic acid and of pyruvic acid to increase. The difference between the two groups of animals is that in the latter the character of the curve after the administration of insulin indicates a more rapid return of glucose to normal levels. The concentration of lactic acid and pyruvic acid, having reached a maximum between 30 and 60 minutes after injection of insulin, also decreases during the last hour in the dogs which received insulin.

DISCUSSION

It has previously been found by Stotz and Bessey (11) and by Klein (5) in the normal and diabetic human being and has been confirmed by us in

normal dogs (6), that lactic acid and pyruvic acid vary together during rest. In the present experiment dogs which were completely depancreatized presented a theoretically satisfactory preparation upon which it was possible to study the action of insulin on the relationship between lactic acid and pyruvic acid. The ratio between these two metabolites was found to remain within a fairly narrow range before and after the injection of insulin following a previous injection of glucose. In conformity with the investigations of Bueding et al. (4), it was found that in the dog which has been depancreatized and received no injections of insulin pyruvic acid remained low after the injection of glucose until insulin was injected, after which there was a great increase. In these animals lactic acid was found to follow exactly the same pattern as pyruvic acid.

In comparison with the slow response of glucose in the depancreatized animals receiving no insulin, the glucose in those which had been maintained in good health with insulin injections fell more rapidly. The rise in the lactic acid and pyruvic acid was shorter lived in these dogs. From previous work it is known that there is little, if any, accumulation of pyruvic acid in the blood after the injection of insulin unless the level of blood sugar is above normal (4, 5). Since the blood sugar level fell following the insulin injection more rapidly in the animals maintained on insulin than in the others, the concentration of pyruvic acid and lactic acid also decreased sooner. As a possible explanation for the better utilization of carbohydrate in the healed animals than in those used postoperatively, it is suggested that the traumatism of the operation and the narcosis, in addition to interferences with water balance and food supply, must influence the liver and the other organs in their functions of glycogen formation and carbohydrate oxidation.

The ratios were calculated between lactic and pyruvic acids for the seven diabetic dogs. As indicated in Tables II and III, the ratios for these dogs remain, with few exceptions, within the range of the ratios for normal dogs, despite the large changes in the concentrations of both metabolites following the injection of insulin. These results reveal that insulin has no direct influence upon the relationship between pyruvic acid and lactic acid.

It should be recognized that the level of lactic acid in the arterial blood represents the mean value resulting from the lactic acid production and removal by the various organs (12). In the same manner the concentration of pyruvic acid in the blood represents the balance between its production and utilization. These results, therefore, show that under the influence of glucose and insulin the rate of formation of these two acids exceeds their removal. It has previously been shown that the diabetic can utilize pyruvic acid (4, 13). It is, therefore, the production which is disturbed in diabetes. The change in concentration in lactic acid and

pyruvic acid occurring after the injection of glucose is small in comparison with the change which occurs after the injection of glucose and insulin. This shows the importance of insulin in the elaboration of lactic acid and pyruvic acid during rest. The probability is that only pyruvic acid is formed as a direct result of insulin, while the concomitant rise in lactic acid occurs indirectly because of an equilibrium between the two metabolites.

In accordance with the Embden-Meyerhof (1) scheme, which is the one accepted at present, it would be necessary for glucose to enter the metabolic path of carbohydrate catabolism by the formation of glucose-6-phosphate. It is well known that after the injection of glucose there is an increase of glucose phosphate in a normal animal; i.e., one capable of forming insulin in response to a high level of glucose. Since Sacks (14) and Kaplan and Greenberg (15) have shown that energy-rich phosphocreatine and adenosine triphosphate can be formed under the stimulus of insulin, it is possible that some of the energy required for the phosphorylation of glucose is acquired from these energy-rich phosphate bonds.

SUMMARY

The injection of insulin into resting depancreatized dogs leads to the accumulation of lactic acid and pyruvic acid. In depancreatized dogs examined 48 to 96 hours postoperatively without receiving insulin injections, and in depancreatized animals used only after recovering from the operation by maintenance with insulin and pancreatin until 18 to 42 hours before the experiment, the responses to the injection of insulin were somewhat different. In the former, the fall in blood sugar was slower and the concentrations of lactic acid and pyruvic acid continued to rise for 2 hours after the injection of insulin, while in the latter, the fall in glucose was more rapid and the concentration of lactic acid and pyruvic acid reached a maximum at 30 to 60 minutes following insulin and then decreased. Despite the large rises in lactic acid and pyruvic acid caused by the injection of insulin, the ratio between the two acids exhibited only small fluctuations.

BIBLIOGRAPHY

1. Meyerhof, O , Biological symposia, Lancaster, 5, 141 (1941).
2. Tolstoi, E , J Biol Chem., 60, 69 (1924).
3. Shorr, E , Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, 7, 323 (1939)
4 Bueding, E , Fazekas, J. F., Herrlich, H , and Himwich, H. E , J. Biol. Chem., 148, 97 (1943).
5. Klein, D , J Biol Chem., 145, 35 (1942)
6. Chesler, A , Homburger, E , and Himwich, H E., J. Biol. Chem., 153, 219 (1944).

7. Friedemann, T. E., and Haugen, G. E , *J Biol. Chem.*, **147**, 415 (1943).
8. Somogyi, M , *J Biol. Chem* , **86**, 655 (1930)
9. Hagedorn, H. C., and Jensen, B N , *Biochem Z* , **135**, 46 (1923).
10. Barker, S. B , and Summerson, W H , *J Biol Chem* , **138**, 535 (1941)
11. Stotz, E., and Bessey, O A , *J. Biol Chem* , **143**, 625 (1942)
12. Himwich, H E , Koskoff, Y D , and Nahum, L H , *J Biol. Chem.*, **85**, 571 (1929–30)
13. Fazekas, J F , Baker, Z , and Himwich, H. E , *Am. J. Physiol.*, **123**, P62 (1938).
14. Sacks, J , *Science*, **98**, 388 (1943)
15. Kaplan, N., and Greenberg, D. M , *J. Biol. Chem* , **150**, 479 (1943).

l-AMINO ACID OXIDASE OF ANIMAL TISSUE

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As early as 1910 Neubauer and his colleagues (1) presented the first experimental evidence from perfusion studies that natural amino acids are oxidized to their corresponding keto acids and ammonia. More recently Krebs (2), using the tissue slice technique, has confirmed and extended the original observation of Neubauer. Yet, despite the growth of a very extensive literature on the metabolism of the individual amino acids, practically no information has been available regarding the nature and number of the enzymes which are involved in their oxidative deamination. Von Euler, Adler, Gunther, and Das (3), and Dewan (4) in 1938 discovered an enzyme in heart muscle and liver which catalyzes the oxidation of $l(+)$-glutamic acid. More recently, Ratner et al. described an enzyme in kidney and liver which catalyzes the oxidation of glycine to glyoxylic acid and ammonia (5). No success has hitherto attended efforts to extract the enzymes or enzyme capable of deaminating oxidatively the rest of the twenty or more natural amino acids. Krebs (2) and others (6) have succeeded in isolating an enzyme in liver and kidney which oxidizes a large number of the amino acids of the d series but which has no effect on the corresponding natural enantiomorphs.

The present communication deals with the properties of an enzyme obtained from rat kidney and liver which catalyzes the oxidation of some thirteen natural amino acids; viz., leucine, methionine, proline, norleucine, norvaline, phenylalanine, tryptophane, isoleucine, tyrosine, valine, histidine, cystine, and alanine. In the presence of oxygen as hydrogen acceptor, 1 molecule of oxygen is taken up for each molecule of amino acid oxidized, and 1 molecule each of keto acid, NH_3, and H_2O_2 are formed.

$$(1) \qquad RCHNH_2COOH + O_2 \rightarrow RCOCOOH + H_2O_2 + NH_3$$
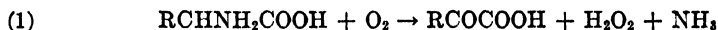
In the presence of catalase H_2O_2 is decomposed into molecular oxygen and the net reaction is therefore

$$(2) \qquad RCHNH_2COOH + \tfrac{1}{2}O_2 \rightarrow RCOCOOH + NH_3 + H_2O$$
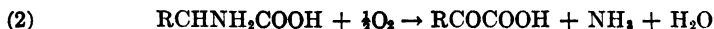
Whether the quantitative relationship will agree more closely with equation (1) or (2) will be determined by the extent to which catalase is present in the enzyme preparation.

With *l*-leucine as substrate, the rate of oxidation under the conditions which apply to equation (2) is practically identical whether molecular oxygen or methylene blue is used as hydrogen acceptor. This identity of rates applies to all substrates of the enzyme except methionine, tryptophane, and tyrosine. In these cases, the rate of reduction of methylene blue is much slower than the rate of reduction of molecular oxygen. The discrepancy in rates is referable to the fact that the resulting keto acids are further oxidized by H_2O_2 formed in the aerobic reaction. As expected, the rates of ammonia formation are also from one-half to one-third the rates of oxygen consumption in these three cases.

For reasons which are not entirely clear to us, only rat kidney and to a lesser extent rat liver have provided a uniformly satisfactory source of active enzyme. A great many alternative sources of the enzyme have been explored with no success whatsoever, including practically all the species available either for experimental purposes or from slaughter-houses (cat, dog, guinea pig, rabbit, pig, ox, and sheep). In one or two instances, we have convinced ourselves that the enzyme is present but at far too low a level of activity to justify further investigation. We have been restricted perforce to rat kidney and liver as experimental material. In the early stages of the present investigation shortage of material proved a severe handicap. However, more recently, thanks to the splendid cooperation of many pharmaceutical companies in collecting large numbers of fresh rat kidneys for us, the problem of inadequate starting material has become less severe. In order to obtain the data discussed below, several thousand rat kidneys were required.

The *l*-amino acid oxidase at least of rat kidney and liver is not associated with insoluble particles. It can readily be prepared in highly purified and soluble form and is reasonably stable.

Preparation of Enzyme—A detailed account of the methods of preparation will be found in the experimental section. Essentially, two alternative procedures were followed. In the first, the kidneys were minced in 15 per cent sodium sulfate and the enzyme purified by successive precipitation at this salt concentration and at pH 4.5. The final solution of the *l*-amino acid oxidase prepared by this method contained no trace of the *d*-amino acid oxidase or glycine oxidase and at most traces of catalase. Most of the experiments reported below were carried out with such a preparation of the enzyme.

The second procedure involved preliminary dehydration of the kidneys or liver with acetone at low temperatures. The preliminary acetone treatment of the kidneys makes it possible to work up rapidly much larger quantities of starting material. However, this advantage, as far as speed of

manipulation is concerned, is partially offset by the fact that crude preparations of the *l*-amino acid oxidase obtained by the second procedure contain some *d*-amino acid oxidase. While the *d* enzyme is rapidly and completely destroyed when rat kidney is minced in 15 per cent sodium sulfate, destruction of the *d* enzyme is arrested by subjecting the kidney to preliminary desiccation with acetone. Presumably there is an agent present in freshly minced rat kidney which inactivates the *d* enzyme. This agent in turn is inactivated by the treatment with acetone. The *l* enzyme is less soluble in salt solutions than the *d* and the ratio of *d:l* activity decreases with each precipitation by 15 per cent sodium sulfate at pH 4.5. Thus the ratio of activity dropped from the initial value of 6.2:1 to 0.25:1 after three precipitations. A 25-fold concentration of *l* enzyme relative to *d* was thus attained in the course of three precipitations. Also, the conditions under which the precipitations were carried out lead to the partial splitting of the *d*-amino acid oxidase, though the *l* enzyme remains unaffected.

Since the relative velocities at which the various amino acids are oxidized by the *d* and *l* enzymes respectively differ widely, the value for the ratio of *d:l* activity will vary depending on which substrate is used for the respective enzymes. In the illustration given above of the change in ratio, the activity of the *l* enzyme was determined by the rate of reduction of methylene blue in the presence of *l*-leucine, while that of the *d* enzyme was determined with *d*-leucine as substrate. In another such experiment *dl*-phenylalanine and *l*-leucine were used to measure *d* and *l* activity respectively. The initial ratio of *d:l* activities was 3.9:1, whereas after three precipitations, the ratio fell to 0.18:1, which represents at least a 20-fold concentration. These ratios actually favor *d* activity, for not only is phenylalanine more rapidly deaminated by the *d* enzyme than the *d*-leucine employed previously, but the estimate of *d* activity was slightly higher than would have been found had *d*- rather than *dl*-phenylalanine been used as substrate. This error cannot, however, be large, since *l*-phenylalanine is attacked at about one-fifth the rate of *l*-leucine. In the final stages of the purification procedure no trace of the *d* enzyme could be found.

Evidence of Complete Elimination of d-Amino Acid Oxidase—When the *l* enzyme was prepared by direct extraction of freshly minced rat kidney with neutral sodium sulfate solution only a trace of *d* activity was found in the first extract. After several precipitations in acidified sodium sulfate solutions, all *d* activity disappeared completely. As shown in Table I, only the *l* forms of phenylalanine, phenylaminobutyric acid, methionine, leucine, proline, N-methylleucine, isoleucine, and tyrosine were found to be oxidized. Had *d*-amino acid oxidase been present, the rates of oxidation in the presence of the *dl*-amino acids would have been higher than those ob-

served for the *l* isomers. As a matter of fact, the rates in the presence of the racemates were slightly lower than those in the presence of the *l* forms. In the case of proline, the rates for *dl* and *l* were almost identical.

The optically active amino acids were either commercial samples isolated from natural sources or were prepared by enzymatic resolutions (see "Experimental"). In either case, the possibility that the results might be affected by small amounts of the *d* form present as a contaminant of the *l* had to be considered. All the optically active amino acids were employed only after they were found to be optically pure when tested both by exposure to purified *d*-amino acid oxidase of pig kidney and by determinations of the optical rotation.

TABLE I

Stereochemical Specificity of l-Amino Acid Oxidase

Substrate	Oxygen uptake		
	l	*d*	*dl*
	c mm	*c mm*	*c mm*
N-Methylleucine	70		62
Phenylaminobutyric acid* ...	214		170
Phenylalanine	161		139
Methionine		0	53
Leucine .	90		57
Isoleucine	119		92
Proline	157		160
Tyrosine*	98		71

The molar concentration of the *dl* form (M/30) was twice that of the *d* or *l* form (M/60). For the amino acids marked with an asterisk, crystals of the *dl* and *l* forms were added directly to the manometer cups The data of this table have no relevance to relative velocities of oxidation, since the duration of the experiment varied from one pair of enantiomorphs to another.

Quantitative Relationship between Oxygen Consumption and Products of Oxidation—According to equation (2), which applies in the presence of excess catalase, the formation of 1 mole of NH_3 and 1 of keto acid should result from the utilization of 1 atom of oxygen. The data of Table II show that this relation is exactly satisfied when the products of oxidation of leucine, norvaline, norleucine, phenylalanine, isoleucine, and N-methylleucine are estimated and compared with the oxygen uptake. Under the same set of experimental conditions much more oxygen is consumed during the oxidation of tyrosine, methionine, and tryptophane than corresponds to the production of ammonia. Instead of 1 atom of oxygen per molecule of ammonia, the results range from about 1.5 atoms in the case of methionine

to about 3 in the case of tryptophane. The explanation of this discrepancy may be found in the marked instability of the keto acids of methionine, tryptophane, and tyrosine in the presence of H_2O_2. Apparently the action of catalase is not sufficiently rapid to protect these keto acids from further oxidation by the peroxide formed in the reaction.

According to equation (1) which applies in the complete absence of catalase, 1 molecule of oxygen should be taken up for each molecule of amino acid oxidized. It has been possible only in one case, *viz.* that of N-methylleucine, to get that precise relationship (*cf.* Table II). In all other cases, the results are intermediary between 1 atom of oxygen and 1 molecule of oxygen. Removal of the last traces of catalase offers considerable technical

<div align="center">TABLE II</div>

Relationship between Oxygen Uptake and Ammonia and Keto Acid Production

Substrate	Addition	Oxygen absorbed	Ammonia formed	Keto acid formed
		microatoms	*micromoles*	*micromoles*
l-Leucine	Excess catalase	29 3	28.8	29.1
dl-Norleucine	" "	22.5	22.7	21.8
dl-Norvaline	" "	15.3	15.0	15.1
dl-Phenylalanine	" "	12.2	11 5	12.0
dl-Isoleucine	" "	7 7	9 8	7.1
l-N-Methylleucine	" "	26 5	23 7	23.0
dl-Methionine	" "	48 5	30.0	
l-Tryptophane	" "	22 6	8.8	
l-Tyrosine	" "	8 4	2.8	
l-N-Methylleucine	None	39.8	21 4	20.1
dl-Norleucine	"	31 6	23 5	15.3
dl-Norvaline	"	32 7	21 3	8.7
dl-Isoleucine	"	15.9	10.7	7.8

Catalase was added in the form of a concentrated and purified preparation from pig kidney   All the above experiments were carried out between pH 7.6 and 8.0.

difficulty in view of the tremendous catalytic activity of catalase. In addition, H_2O_2 may be decomposed by other constituents of the enzyme solution such as heavy metals, proteins, etc. The instability of many α-keto acids in the presence of H_2O_2 introduces still another factor which militates against satisfying equation (1). The yield of keto acid will, therefore, invariably be lower than that of ammonia.

The 2,4-dinitrophenylhydrazones of the keto acids corresponding to methionine, leucine, norleucine, and proline have been isolated in good yield and identified. That of proline appears to be identical with the hydrazone of α-keto-δ-aminovaleric acid which was reported by Krebs (7) as the product of the action of *d*-amino acid oxidase on *d*-proline.

Further Evidence of H_2O_2 Formation—The detection of such small amounts of H_2O_2 as are formed during oxidative deamination is complicated by the decomposition of peroxide due to traces of catalase or to further oxidation of keto acid. Keilin and Hartree (8) circumvented direct estimation by introducing a reaction involving oxidation of ethyl alcohol by H_2O_2, which proceeds more rapidly than the catalytic decomposition of H_2O_2. If H_2O_2 is actually formed in the reaction involving the *l* enzyme, the effect of adding alcohol to the system composed of *l*-amino acid oxidase, substrate, and excess catalase will be to change the ratio of atoms of oxygen consumed to moles of NH_3 produced from 1:1 to 2:1. This method of detecting

TABLE III

Effect of Ethyl Alcohol on Oxygen Consumption and Ammonia Production of System l-Amino Acid Oxidase, Substrate, Catalase

	Oxygen uptake		Ammonia formation	
	Without alcohol	With alcohol	Without alcohol	With alcohol
	atoms	*atoms*	*moles*	*moles*
l-Leucine	9 5	18 2	9.5	8.8
	11 5	23 1		
	5 1	11 6	6 0	7 5
l-N-Methylleucine	10 5	19 7		
	11 1	18 2	12.4	12.4
dl-Aminocaproic acid	13 1	19 1	13 3	10 2
l-Proline	11 5	22 2		
dl-Aminovaleric acid	18 9	37 0	17.2	16.2

The following additions were made 1 cc of *l*-amino acid oxidase, 0 5 cc. of 0.1 M amino acid, 0 3 cc of concentrated catalase solution prepared from pig kidney, and 0 1 cc of 95 per cent alcohol Final volume 3 cc , 38° The solutions of the two enzymes and the substrate were adjusted to pH 9 0 with dilute alkali

H_2O_2 formation was applied repeatedly and always led to an increase in oxygen consumption without corresponding increase of NH_3 production (*cf.* Table III).

Washed red blood cells have occasionally been used as a source of catalase with which to supplement catalase-poor preparations of the *l*-amino acid oxidase for experiments on oxygen uptake. During these runs the red color of hemoglobin was replaced by the brown color of methemoglobin. This oxidation of hemoglobin did not take place in the absence of amino acid and is undoubtedly attributable to H_2O_2 formed during the enzymatic oxidation of amino acids by molecular oxygen.

Specificity of l-Amino Acid Oxidase—The enzyme acts on all of the naturally occurring monoaminomonocarboxylic acids except glycine, threo-

nine, and serine. The dicarboxylic acids, glutamic and aspartic acids, are not attacked nor are the dibasic amino acids, lysine, ornithine, and arginine. Among the sulfur-containing amino acids methionine is oxidized rapidly and cystine very slowly. The rapid non-enzymatic oxidation of cysteine by oxygen or methylene blue makes it difficult to determine whether the enzyme oxidizes cysteine. Some sulfur-substituted derivatives of cysteine are attacked readily, e.g. S-benzylcysteine and S-ethylcysteine, but S-carboxymethylcysteine is oxidized very slowly. The secondary amino acid, proline, is oxidized quite rapidly as are all the N-monomethyl-amino acids that have been tested; viz., those of leucine, methionine, homocysteine, and S-benzylhomocysteine.

TABLE IV

Relationship between Chain Length and Rate of Oxidation

Substrate	No of carbon atoms	Oxygen uptake	
		0 017 M	0 0056 M
		c mm	c mm
Glycine	2	0	
dl-Alanine	3	3	
dl-Aminobutyric acid	4	11	
dl-Aminovaleric "	5	20	
dl-Aminocaproic "	6	46	25
dl-Aminocaprylic "	8	35*	19

* Aminocaprylic acid was not sufficiently soluble to test at 0 017 M concentration. The velocity of aminocaprylic acid at 0.017 M was calculated from its relative velocity at 0.0056 M

Table IV shows the relationship between velocity of oxidation and the number of carbon atoms in the series of straight chain amino acids. Maximum velocity is attained at a chain length of 6 carbon atoms with a falling off below and above this number. The insolubility of higher homologues makes it virtually impossible to test the relationship beyond aminocaprylic acid.

Mechanism of Reaction—Thus far there is no reason to consider the mechanism of oxidation as other than the removal of 2 hydrogen atoms to form the imino acid as postulated for other amino acid-oxidizing enzymes. In accordance with this mechanism, N-monomethylleucine in the presence of the oxidase yielded the corresponding keto acid and methylamine. The latter was isolated and identified in the form of its picrolonate. The dehydrogenation of N-methylleucine by an alternative pathway to yield leucine and formaldehyde, though theoretically possible, does not occur. Thus, both l-amino acid oxidase and glycine oxidase (5) are completely analogous

in their action on N-methylamino acids.   N-Dimethylleucine and α-amino-isobutyric acid were not attacked, presumably since imino acid formation is precluded.   In the case of proline, no ammonia is liberated during the oxidative reaction but the ring is opened with formation of both a carbonyl and a primary amino group.   The resulting compound reacts with sulfite, contains a free amino group, and forms a 2,4-dinitrophenylhydrazone which, like dinitrophenylhydrazones of other α-keto acids, gives a reddish brown color in strong alkali, and contains a free amino group.   Ring cleavage therefore appears to take place exclusively between the N and the carbon atoms of the pyrrolidine ring.

TABLE V ·

Relative Velocities of Oxidation of Amino Acids by l-Amino Acid Oxidase of Rat Kidney

l-N-Methylleucine	115	*dl*-Phenylalanine	45
l-Leucine	100	*l*-Tryptophane	40
dl-Aminocaproic acid .	89	*dl*-Valine	28
dl-Methionine	81	*l*-Tyrosine*	20
l-Proline	77	*l*-Cystine* . .	15
dl-Isoleucine	71	*l*-Histidine	9
dl-Aminovaleric acid	53	*dl*-Aminobutyric acid	3

All velocities are expressed as percentages of the velocity of *l*-leucine taken as 100.   The experiments were carried out at 38°.   The final concentration of the substrate was M/60 except for the amino acids marked with an asterisk, the solubility of which was not adequate to permit a final concentration of M/60.   In these two instances, the relative velocities would be appreciably higher when corrected for the effect of concentration   Rate of ammonia production was used as a direct measure of the velocity of oxidation   In the case of proline, the rate of oxygen uptake was taken as a measure of the velocity of oxidation, since no ammonia is formed during the reaction.

Number of Enzymes—The available evidence is consistent with the assumption that only one enzyme catalyzes the oxidation of the various amino acids listed in Table V in the order of their rates of oxidation.   Regardless of the state of purity of the enzyme from the crudest material to the most highly purified enzyme, the list of amino acids which are attacked does not change and the relative rates of oxidation do not undergo significant change (*cf.* Table VI).   We have observed minor variations in the order of activities from one preparation to another.   Most if not all of the variation is related to the difficulty of pH control.   The velocity of oxidation is greatly dependent upon the hydrogen ion concentration and a difference of 0.1 to 0.2 pH unit affects the velocity appreciably (*cf.* Fig. 3).   Since each amino acid and each preparation of the enzyme represents a different buffer system, it is clearly not a simple task to insure that the final pH of the system

TABLE VI

Order of Velocities of Oxidation of Amino Acids by Various Preparations of l-Amino Acid Oxidase

The velocities are arranged in order from 1 to 11 with the highest velocity first.

Source of enzyme from rat...	Kidney	Kidney	Liver	Kidney	Kidney	Kidney	Kidney
Method of preparation*...	A	A	B	C	C	D	D
Method of testing	Methylene blue	O_2 uptake	O_2 uptake	O_2 uptake	NH_3 formation	O_2 uptake	NH_3 formation
1	N-Methylleucine	N-Methylleucine	N-Methylleucine	Aminocaproic	N-Methylleucine	N-Methylleucine	N-Methylleucine
2	Leucine	Leucine	Leucine	N-Methylleucine	Leucine	Phenylalanine	Leucine
3	Aminocaproic	Aminocaproic	Phenylalanine	Leucine	Aminocaproic	Leucine	Aminocaproic
4	Phenylalanine	Phenylalanine	Aminocaproic	Phenylalanine	Isoleucine	Isoleucine	Isoleucine
5	Aminovaleric	Proline	Isoleucine	Proline	Aminovaleric	Aminocaproic	Aminovaleric
6	Isoleucine	Aminovaleric	Proline	Aminovaleric	Phenylalanine	Aminovaleric	Phenylalanine
7	Valine	Isoleucine	Aminovaleric	Isoleucine	Valine	Proline	Valine
8	Histidine	Valine	Valine	Histidine	Cystine	Valine	Histidine
9	Alanine	Alanine	Cystine	Cystine	Histidine	Histidine	Alanine
10	Cystine	Cystine	Alanine			Alanine	Cystine
11			Histidine			Cystine	
Amino acids omitted	Proline, cystine	Histidine		Alanine, valine	Alanine, proline		Proline

Tyrosine, tryptophane, and methionine were not included in the comparison since the rates of oxygen uptakes in the presence of these amino acids are not equivalent to the rates of ammonia production. They were oxidized by all the preparations.

* The letters indicate the following methods of preparation: A, mincing with 15 per cent Na_2SO_4, followed by three acid precipitations; B, acetone desiccation, two acid precipitations with Na_2SO_4, 10 minutes heating at 55°; C, acetone desiccation, two acid precipitations, dialysis, adsorption on and elution from alumina C_γ; D, as for C with final ammonium sulfate fractionation following which the enzyme is about 50 per cent pure.

amino acid, enzyme, and buffer will be the same from one amino acid and from one series to another. The buffering effect of each amino acid can readily be corrected for provided an adequate supply of enzyme solution is available for preliminary pH measurements. Owing to limitations of material this condition could not be fulfilled. While the pH control was not sufficiently adequate (control within 0.1 pH unit) to eliminate minor variations in the order of activities, it was adequate for the main purpose of these experiments, which was to show that the same amino acids are attacked and at substantially the same velocities in preparations widely different in the level of purity, method of preparation, and source. These results are incompatible with the assumption that more than one enzyme is involved. Another confirmatory bit of evidence is the fact that the *l*-amino acid

TABLE VII

Summation of Oxidation of Pairs of Amino Acids

Substrate	Oxygen uptake	Expected increase in oxygen uptake for perfect summation	Observed increase in oxygen uptake	Summation
	c mm	*c mm*	*c mm*	*per cent*
l-Leucine	201			
l-Proline	116			
l-N-Methylleucine	342			
l-Ethylcysteine	371			
l-Leucine + *l*-proline	187	116	0	0
" + *l*-N-methylleucine	350	201	8	4
" + *l*-ethylcysteine	318	201	0	0

The above experiments were carried out over a period of 5 hours at 38°. The final concentration of each substrate was M/60 in every case

oxidase of rat liver attacks the same amino acids as its counterpart in rat kidney and at materially the same rates (*cf.* Table VI). Were more than one enzyme involved in the oxidation of the thirteen odd amino acids, it would be all but impossible to find such a parallelism in mixtures of enzymes from two entirely different sources. Finally, no summation of rates was observed when various pairs of amino acids were tested as substrates. The rates were either the same as or slightly less than the rate of the more rapidly oxidized of the pair of amino acids (Table VII).

General Properties of l-Amino Acid Oxidase—The enzyme has the pleasing property of maintaining the initial velocity of oxidation practically unchanged over long periods of time (*cf.* Fig. 1). In the early phases of the investigation, when the level of activity was very low, the ability of the enzyme to carry on over a period of 8 hours without serious loss of activity made it possible to do many more experiments than the initial activity ordi-

narily would warrant.   Figs. 2 to 5 summarize the effects of varying the
concentration of substrate, concentration of hydrogen ion, concentration
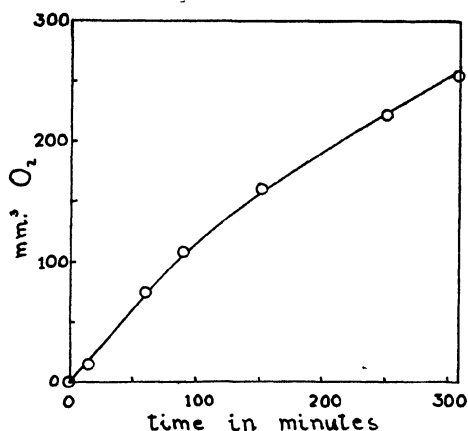
FIG. 1. Time-activity curve of l-amino acid oxidase of rat kidney.   The manometer
cup contained 2 cc. of enzyme, 0.5 cc. of 0.1 M l-leucine, 0.9 cc. of 0.25 M dimethylgly-
cine buffer of pH 8 2, and 0.1 cc. of a washed suspension of sheep red blood corpuscles
as source of catalase.   Final volume 3.5 cc.; 38°; alkali in center well.
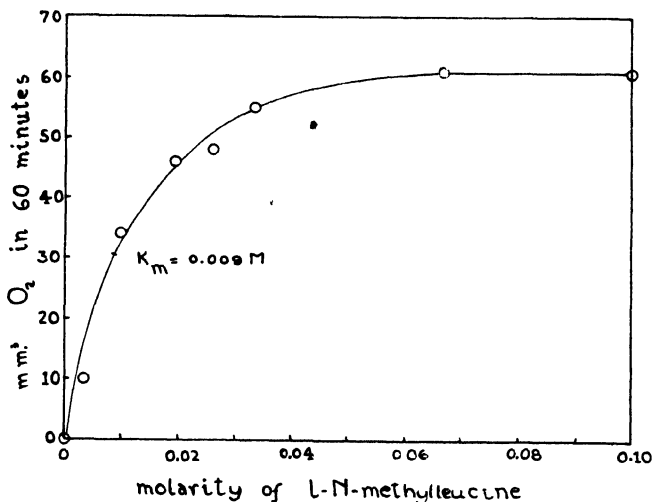
FIG. 2. Rate of oxygen uptake as a function of the concentration of l-N-methylleu-
cine.   Each manometer cup contained 1.5 cc. of enzyme, buffered at pH 8.2.   Final
volume 3 cc.; 38°; alkali in center well.

of enzyme, and the temperature on the rate of oxygen uptake.   Figs. 2, 4,
and 5 are self-explanatory and need no further comment.   In Fig. 3,
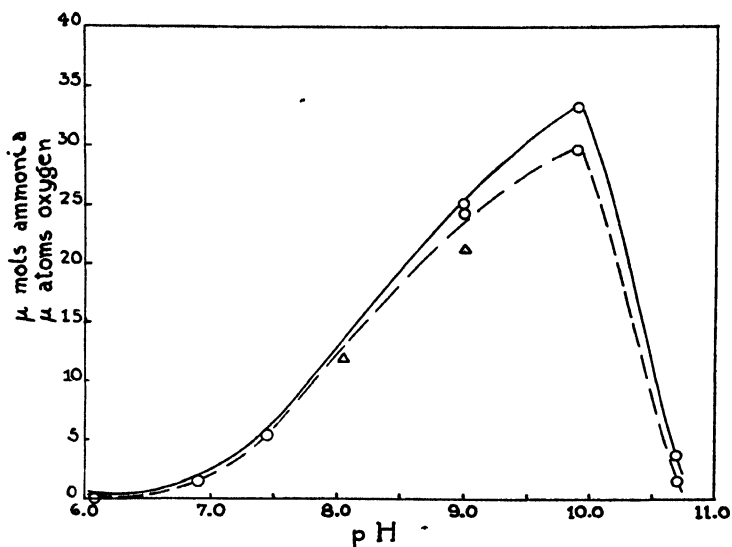
FIG. 3. Rates of oxygen uptake and ammonia production as a function of pH. Each manometer cup contained 1 cc of dialyzed enzyme solution, 0.5 cc. of 0.1 M l-N-methylleucine, 0.5 cc. of 0.25 M buffer, and 0.5 cc. of dialyzed catalase solution. Final volume 3 cc.; 38°; alkali in center well. The following symbols indicate the nature of the buffer: ○ phosphate, △ dimethylglycine. The continuous curve represents oxygen uptake and the broken curve represents ammonia formation. The pH values were final values, determined electrometrically.
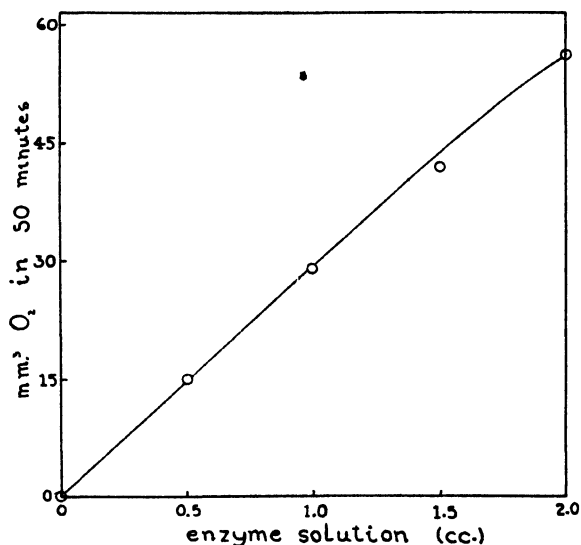
FIG. 4. Rate of oxygen uptake as a function of the concentration of enzyme. Each manometer cup contained 1 cc. of 0.1 M l-N-methylleucine in a final volume of 3 cc. Alkali in center well; 38°; air in gas space.

velocity of oxidation was measured both by ammonia formation and by oxygen uptake. The pH maxima are identical at about 10. Above and below the maximum pH the velocity of oxidation declines sharply. It is of considerable interest that the rate of oxidation at pH 7 is about one-tenth the maximum velocity. Below pH 7 the velocity of oxidation is negligible.
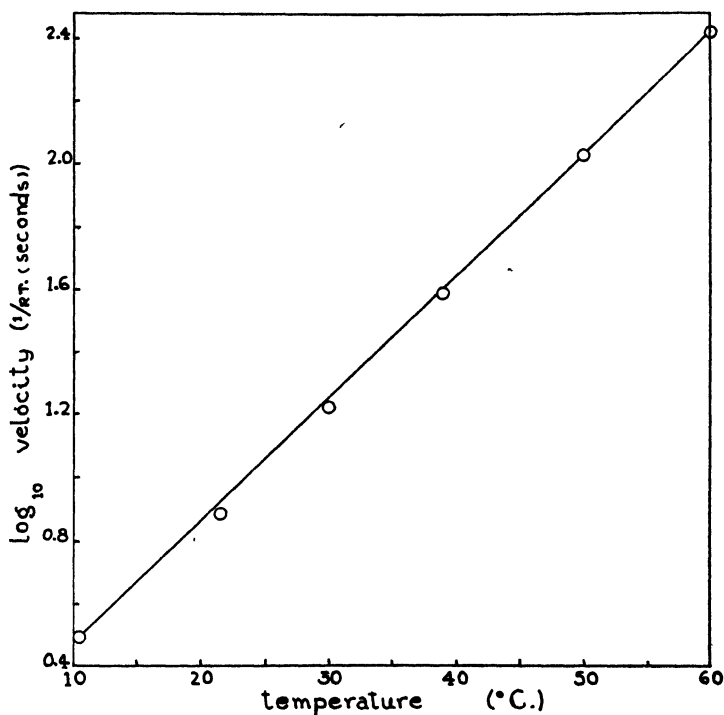
FIG. 5. Rate of methylene blue reduction as a function of the temperature. Each Thunberg vessel contained 1.5 cc. of enzyme buffered at pH 10 and 0.5 cc. of *l*-N-methylleucine in the main tube, and 0.1 cc. of 0.1 per cent methylene blue in the hollow stopper. The contents of the evacuated tubes were mixed after complete temperature equilibration.

Table VIII summarizes the effects of various inhibitors on the activity of *l*-amino acid oxidase. Benzoic acid which inhibits completely the action of *d*-amino acid oxidase (Handler and Bernheim (9)) and glycine oxidase (5) in 0.001 M concentration has practically no influence on the *l*-oxidase even at 0.01 M concentration. 0.01 M KCN which inhibits the aerobic oxidation of *l*-amino acids by the oxidase of *Proteus vulgaris* (10) has no effect on the *l* enzyme of rat kidney either aerobically or anaerobically. Similarly, capryl alcohol which completely inhibits the action of the *l* enzyme from

Proteus vulgaris has no effect on the *l* enzyme from rat kidney. An inhibition which is peculiar to the *l*-oxidase of kidney is that by ammonium ions.

The oxidase survives exposure to 60° for 5 minutes without appreciable loss of activity. This fortunate property has been taken advantage of in the preparation in order to eliminate impurities by heat denaturation. Exposure to 70° for 5 minutes completely destroys the oxidase.

Nature of Prosthetic Group—With progressive purification and concentration the enzyme solution becomes more highly colored. The details of the method of purification will be dealt with in another communication. Our best preparations are intensely yellow with a greenish fluorescence.

TABLE VIII

Inhibitors of l-Amino Acid Oxidase

Inhibitor	Final concentration	Inhibition	Method of test
	M	*per cent*	
Ammonium sulfate	0 12	100	Thunberg
" "	0.066	96	"
" "	0.055	79	"
" "	0 012	69	"
Capryl alcohol	Saturated solution	0	" and manometric
Zinc sulfate	0 001	5	"
Copper sulfate	0 001	43	"
Iodoacetic acid	0 001	71	"
Arsenious "	0 01	0	Manometric
Sodium fluoride	0 01	0	"
Malonic acid	0 01	0	"
Methyltestosterone	Saturated solution	0	"
α-Aminoisobutyric acid	0 017	0	"
Benzoic acid	0 01	28	"
" "	0 001	13	"
Potassium cyanide	0 002	0	" and Thunberg

The color is bleached on addition of $Na_2S_2O_4$ and restored by shaking with air. From data obtained with the ultracentrifuge and the Tiselius apparatus it appears that all the color is associated with a single protein component. On addition of *l*-leucine under anaerobic conditions, the yellow color is rapidly bleached just as by $Na_2S_2O_4$. Admission of air restores the original color. This strongly suggests that the *l* enzyme is a chromoprotein with the colored prosthetic group undergoing cyclical reduction by the substrate and oxidation by molecular oxygen.

Tests for the presence of flavoprotein enzymes such as xanthine oxidase, aldehyde oxidase, *d*-amino acid oxidase, and glycine oxidase were negative. These flavoproteins can therefore be excluded from consideration. How-

ever, the presence of a diaphorase in the preparation of the *l*-amino acid oxidase has been consistently detected in all stages of purity. The constancy of the ratio of diaphorase activity to *l*-amino acid oxidase activity suggests that the two enzymes are associated with the same protein in a way comparable to the chromoprotein of milk which carries three catalytic activities; *viz.*, those towards purine, aldehyde, and dihydrocoenzyme I respectively (11).

<div align="center">EXPERIMENTAL</div>

Preparation of Enzyme—Rat kidney and liver were obtained fresh from slaughtered animals and kept frozen on dry ice until required. There was no evidence of deterioration of the enzyme in tissues which had been kept frozen for several months. 500 gm. of rat kidney had to be processed to obtain about 75 cc. of enzyme solution, 1 cc. of which takes up about 60 c.mm. of O_2 per hour in the presence of *l*-leucine. The frozen kidneys were thawed out, divested of the connective tissue covering and adhering fat, and minced in a Waring blendor in 14 volumes of 15 per cent sodium sulfate. The fine suspension was acidified with 10 per cent acetic acid to pH 4.5 and centrifuged with hard packing. The supernatant fluid was discarded and the precipitate suspended in half the original volume. Trisodium phosphate solution (0.5 M) was added to bring the pH to about 8.2. The turbid suspension was heated to 55° and maintained for 5 minutes at that temperature. The coagulated protein was readily filtered off. Sodium sulfate (15 gm. per 100 cc.) was added to the clear yellow-brown fluorescent filtrate and the pH brought to 4.4 by addition of 10 per cent acetic acid. The precipitate was centrifuged down and resuspended in the smallest possible volume of water. Trisodium phosphate (0.5 M) was added to make the suspension alkaline (pH 8.2) and the insoluble material centrifuged off and discarded. For routine experiments, the enzyme was used at this stage of purity.

The above method was not applicable to the preparation of *l*-amino acid oxidase from rat liver nor to the large scale preparation of the enzyme from rat kidney. For these purposes, the frozen tissues were thawed, minced finely in a Waring blendor, and poured into 3 volumes of acetone kept at −10° with dry ice. The tissue was filtered off and washed with acetone on the suction filter. It was then dried and pulverized. To remove the last traces of fat the powder was resuspended in anhydrous acetone at room temperature, and then worked up again into a fine powder. About 150 gm. of acetone powder yield as much enzyme as do 500 gm. of kidney in the above method. The powder was suspended in 10 volumes of water and the mixture filtered. The clear red filtrate was then treated with sodium sulfate (15 gm. per 100 cc.). From then on the procedure is identical with that of the above method at the corresponding stage.

Procedures for Isolation and Estimation of Products of Oxidation

2,4-Dinitrophenylhydrazone of α-Keto-γ-methiobutyric Acid—Since all the keto acid derivatives described below were isolated by the same procedure, the following conditions may be regarded as being typical. 35 cc. of a thrice precipitated, catalase-free, enzyme solution were adjusted to pH 8.3 with a few drops of 0.5 M alkaline phosphate solution and aerated for 8 hours at 38° in the presence of 35 cc. of 0.1 M *dl*-methionine. According to the oxygen consumption in a pilot run 23.5 mg. of keto acid should have been formed, which corresponds to a maximum yield of 61 mg. of 2,4-dinitrophenylhydrazone derivative. On completion of the reaction, sufficient strong HCl was added to make a final concentration of 2 N HCl, the solution was filtered, and 160 mg. of 2,4-dinitrophenylhydrazine dissolved in 8 cc. of hot 2 N HCl were added. The mixture was chilled overnight; the crystals were filtered off, dried, and recrystallized three times from ethyl acetate by the addition of petroleum ether. Yield 43 mg.; m.p. 148°, with decomposition.

Analysis—$C_{11}H_{12}N_4O_6S$ Calculated, C 40 2, H 3 68; found, C 40 2, H 3 75

2,4-Dinitrophenylhydrazone of α-Ketonorcaproic Acid—Yield 30 mg., 80 per cent; m.p. 142–143°, with decomposition, uncorrected. A melting point of 148° has been reported for this compound (12) but we have observed variations with different samples. One preparation which melted with decomposition at 134–135° at the time of isolation (10), and was analytically pure, gave a melting point of 136–137° after standing for several months. Evidently, it was a mixture of two forms (13) and identification by mixed melting point was therefore considered unreliable. The sample with a melting point of 142–143° also gave satisfactory analytical figures.

Analysis—$C_{12}H_{14}N_4O_6$ Calculated, C 46.5, H 4 55; found, C 46.3, H 4 36

2,4-Dinitrophenylhydrazone of α-Ketoisocaproic Acid—Yield 15 mg., 70 per cent; m.p. 155–156°, with decomposition, uncorrected. The mixed melting point with an authentic sample gave no depression.

Analysis—$C_{12}H_{14}N_4O_6$. Calculated, C 46 5, H 4.55; found, C 45 9, H 4.73

2,4-Dinitrophenylhydrazone of α-Keto-δ-aminovaleric Acid—Yield 33 mg., 90 per cent. This compound was obtained as the hydrochloride described by Krebs (7). It was recrystallized three times from 15 per cent HCl, washed with acid of the same concentration, and dried *in vacuo* over KOH and P_2O_5.

Analysis—$C_{11}H_{13}N_5O_6 \cdot HCl$. Calculated, C 38 00, H 4.03; found, C 38.01, H 3 99

The melting point, with decomposition, uncorrected, was 219°. The mixed melting point with a sample prepared by the action of *d*-amino acid oxidase

and which had a melting point of 219° gave no depression. Krebs (7) reports 232–242°.

Isolation of Methylamine from Enzymatic Deamination of l-N-Methylleucine—34 cc. of enzyme solution and 19 cc. of 0.1 M l-N-methylleucine were incubated aerobically at 38° for several hours. Oxygen consumption as determined in a pilot run was equivalent to the formation of 14.6 mg. of methylamine. After removal of protein by the addition of metaphosphoric acid to make a final concentration of 1.2 M, alkali was added to the filtrate and methylamine distilled off into dilute acid. Traces of ammonia arising from hydrolysis of protein amide groups were removed with 2 gm. of HgO in borate buffer under the conditions employed by Rechenberger (14). Methylamine was estimated in a small aliquot and 12.0 mg. found to be present. The base was again distilled into an aqueous suspension containing an exact equivalent of picrolonic acid (100 mg.). After the distillate was concentrated, 92.5 mg. of methylamine picrolonate were obtained which, following two recrystallizations from 95 per cent ethyl alcohol, melted with decomposition at 239°, uncorrected. No depression was observed when mixed with an authentic sample.

Analysis—$C_{11}H_{11}O_5N_5$ Calculated, N 23 7; found, 23.4

Estimation of Keto Acid—The contents of each manometer cup, usually 3 cc., were made up to a volume of 10 cc. with 2 M metaphosphoric acid and filtered. Keto acid was estimated in 1 or 2 cc. aliquots of the filtrate by a micro adaptation of the sulfite-binding method of Clift and Cook (15).

An unsatisfactory feature of this method, when applied to keto acids other than pyruvic, is the failure to bind an exact equivalent of bisulfite (16). This is undoubtedly due to partial dissociation of the sulfite addition compound. The difficulty was overcome by determining the necessary correction factor for each of the keto acids to be estimated. For lack of pure samples of the keto acid in question, various dl-amino acids were subjected to oxidative deamination by d-amino oxidase at pH 7.6 in the presence of catalase. The theoretical iodine titration value was established by agreement between oxygen consumption and ammonia formation. The correction factor was then determined by calculation based on the ratio of the theoretical iodine titration value to the observed one.

The amount of excess bisulfite influences the dissociation of the addition compound and consequently the reproducibility of the correction factor. Uniform conditions were maintained by adding an 80- to 100-fold excess of bisulfite to the sample which was then allowed to stand 15 minutes in stoppered 50 cc. Erlenmeyer flasks. Before titration the pH of the metaphosphoric acid filtrate (1.2 M) was about 2. The first end-point fades rather rapidly in the cases of α-keto-β-methylvaleric acid and of α-ketocaproic acid,

while the second end-point is unstable for phenylpyruvic acid.   The factors obtained were α-ketovaleric acid 1.04, α-keto-β-methylvaleric acid 1.42, pyruvic acid 1.02, α-ketocaproic acid 1.10, α-phenylpyruvic acid 1.12, and α-ketoisocaproic acid 1.15.   In all cases the enzyme blanks contained insignificant amounts of keto acid.

Estimation of Ammonia—1 or 2 cc. aliquots of the metaphosphoric acid filtrate were used for estimation of ammonia by the micro aeration method of Sobel[1] or by micro-Kjeldahl distillation.   The volatile base was collected in boric acid and titrated with N/70 HCl in the micro-Scholander burette (17).   Loss of NH_3, when formed enzymatically in solutions more alkaline than pH 8, was prevented by inserting 2 M metaphosphoric acid in the side arm of the Warburg cup.   A small amount of NH_3 is always found in the blanks even after prolonged dialysis of the enzyme solution.   It undoubtedly arises from hydrolysis of amide linkages of the protein during incubation.   All ammonia values are corrected for the blank which was usually found to be approximately 2 to 3 micromoles.

Preparation of Amino Acids—*dl*-N-Dimethylleucine, S-ethyl-*l*-cysteine, and S-benzyl-*l*-cysteine were prepared by the usual procedures.   *d*-Methionine was made by enzymatic resolution, *Proteus vulgaris* being used to remove the natural component (10).

We are indebted to Dr. V. du Vigneaud for the N-monomethyl derivatives of *dl*-methionine, *dl*-homocysteine, and S-benzyl-*dl*-homocysteine, to Dr. K. Bloch for preparations of *l*- and *d*-phenylaminobutyric acid, and to Dr. M. Stetten for *dl*-proline.

Synthesis of l-N-Methylleucine—This compound was prepared by the methylation of *p*-toluenesulfonyl-*l*-leucine, followed by hydrolytic removal of the toluenesulfonyl group.   The method of synthesis was essentially like that of Fischer and Lipschitz (18) except that dimethyl sulfate instead of methyl iodide was used as the methylating agent.   25 gm. (0.088 M) of *p*-toluenesulfonyl-*l*-leucine were suspended in 100 cc. of water and brought into solution by the addition of 75 cc. of 2 N NaOH.   21.4 cc. of dimethyl sulfate (0.23 M) were slowly added with continuous stirring for 2 hours, during which period 65 cc. of 2 N NaOH were required to maintain an alkaline pH.   Stirring was continued for another hour.   The methyl ester of *p*-toluenesulfonyl-*l*-N-methylleucine separated out as an oil but soon crystallized.   It was recrystallized from hot 50 per cent ethyl alcohol.   Yield 85 per cent; m.p. 75–76°, uncorrected.

$$C_{15}H_{23}O_4NS \quad \text{Calculated, N (Kjeldahl) 4.47; found, 4.50}$$
$$[\alpha]_D^{24} = -31.6° \text{ (3.3\% in 95\% ethyl alcohol)}$$
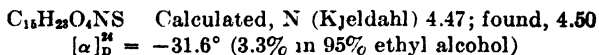
17 gm. of the methyl ester were mixed with 42 cc. of concentrated HCl and 60 cc. of glacial acetic acid and heated for 24 hours at 100° in sealed tubes.

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[1] Sobel, E., personal communication.

The mixture was then concentrated to a syrup *in vacuo* to remove excess acid. The residue was taken up in water and extracted with ether. The aqueous layer was concentrated to 12 cc., neutralized with pyridine, and treated with 3 volumes of alcohol. *l*-N-Methylleucine crystallized out on chilling and was recrystallized from aqueous acetone. The yield after two recrystallizations was 31 per cent. N (Kjeldahl), found 9.35 per cent; theory 9.41 per cent. $[\alpha]_D^{24} = +20.1°$ (3.5 per cent in H_2O). 20 per cent of the starting material remained unhydrolyzed under the above conditions but to avoid racemization longer heating was considered inadvisable.

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SUMMARY

The preparation and some of the properties of the *l*-amino acid oxidase of rat kidney and liver are described. The enzyme catalyzes the oxidation of the *l* forms of leucine, phenylalanine, norleucine, norvaline, isoleucine, valine, cystine, histidine, tyrosine, methionine, alanine, and tryptophane to their corresponding keto acids and ammonia. N-Methylleucine is oxidized to the corresponding keto acid and methylamine. In the case of proline, the pyrrolidine ring is split open oxidatively with formation of α-amino-δ-ketovaleric acid without liberation of ammonia. In the catalyzed reaction of amino acids with molecular oxygen, 1 molecule of oxygen is taken up for each molecule of amino acid oxidized with formation of 1 molecule each of keto acid, ammonia, and hydrogen peroxide. In the presence of catalase only 1 atom of oxygen is taken up for each molecule of amino acid oxidized. Methylene blue can replace oxygen as hydrogen acceptor. The available evidence is consistent with the assumption that only one enzyme catalyzes the oxidation of the thirteen *l*-amino acids. The separation of *l*- from *d*-amino acid oxidase is readily effected. Thus far rat kidney and liver have been found to be the only satisfactory sources of the *l* enzyme.

BIBLIOGRAPHY

1 Neubauer, O , and Gross, W , *Z. physiol Chem* , **67**, 219 (1910)   Neubauer, O , and Fischer, H., *Z. physiol. Chem.*, **67**, 230 (1910).
2. Krebs, H. A , *Biochem. J.*, **29**, 1620 (1935).
3. von Euler, H., Adler, E., Gunther, G., and Das, N. B., *Z. physiol Chem* , **254**, 61 (1938).
4. Dewan, J G., *Biochcm J.*, **32**, 1378 (1938)
5. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem* , **152**, 119 (1944).

6. Straub, F. B., *Nature*, **141**, 603 (1938). Warburg, O., and Christian, W., *Biochem. Z.*, **295**, 261 (1938); **298**, 150 (1938).

7. Krebs, H. A., *Enzymologia*, **7**, 53 (1939).

8. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 141 (1936).

9. Handler, P., and Bernheim, M. L. C., *J. Biol. Chem.*, **150**, 335 (1943).

10. Stumpf, P. K., and Green, D. E., *J. Biol. Chem.*, **153**, 387 (1944).

11. Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, **33**, 1694 (1939).

12. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933). Felix, K., and Zorn, K., *Z. physiol. Chem.*, **258**, 16 (1939).

13. Dirschl, W., and Nahm, H., *Ber. chem. Ges.*, **73 B**, 448 (1940).

14. Rechenberger, J., *Z. physiol. Chem.*, **265**, 222 (1940).

15. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).

16. Long, C., *Biochem. J.*, **36**, 807 (1942).

17. Scholander, P. F., Edwards, G. A., and Irving, L., *J. Biol. Chem.*, **148**, 495 (1943).

18. Fischer, E., and Lipschitz, W., *Ber. chem. Ges.*, **48**, 360 (1915).

A STUDY OF THE VARIOUS PROCEDURES FOR THE ESTIMATION OF TRYPTOPHANE

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As early as 1928 there was occasion to study the tryptophane content of grain curd casein after treatment with acetic acid, alcohol, and ether and drying at a temperature of 85°. Comparison of the treated casein with the original material showed that the cystine and tryptophane content had apparently decreased. The procedure employed for estimating the tryptophane was that of May and Rose (1) as modified by Holm and Greenbank (2).

Since this procedure required from 8 to 10 days for a determination, a modification was introduced in order to shorten the time required. In this modification, the May and Rose (1) procedure was carried out at 85° for 15 minutes and the color development was accelerated by the addition of 0.2 cc. of a 0.3 per cent solution of hydrogen peroxide.

Subsequently Sullivan, Milone, and Everitt (3) made a comparison of the short method with hydrogen peroxide as accelerator with the long May and Rose procedure. By comparing the apparently stable color given by the tryptophane standard in the May and Rose procedure with that given by casein, they concluded that casein contained 2.4 per cent tryptophane and that the short and the long procedures gave the same blue color with casein qualitatively and quantitatively. In the short method at elevated temperature, tryptophane cannot be used as a standard because of the destructive action of the peroxide.

However, at the completion of the work of Sullivan, Milone, and Everitt, Bates (4) reported a modification of the May and Rose procedure whereby, with sodium nitrate as an accelerator, the tryptophane content of proteins can be determined very speedily at room temperature. Employing Bates' procedure on casein, we found a tryptophane content of 1.25 per cent. This value was obtained by the use of a cobalt blue filter to offset the more or less reddish tone of the tryptophane standard. We were doubtful of the validity of the low results with the Bates method not only because of the use of the filter but also because higher values had been reported in the literature both by isolation and by colorimetric estimation.

Tryptophane reacts with various aldehydes to give colored complexes on mild oxidation. Using formaldehyde as a condensing agent, Fürth and Nobel (5) found 1.94 to 2.25 per cent tryptophane in casein, and Fürth and

Lieben (6) found 2 per cent. Using *p*-dimethylaminobenzaldehyde, Thomas (7) found 1.7 per cent, Holm and Greenbank (2) 2.24 per cent, Komm and Böhringer (8) 2.3 per cent, and Jones, Gersdorff, and Moeller (9) 2.2 per cent. By isolation, Hopkins and Cole (10) obtained 1.5 per cent and Dakin (11), after isolation by butyl alcohol extraction, 1.7 per cent.

On the other hand, a number of investigators have reported low tryptophane values in casein. Thus Fasal (12) reported 0.65 per cent, Herzfeld (13) 0.5 per cent, Homer (14) 0.99 to 1.59 per cent, and Lüscher (15) 1.1 per cent. More recently Virtanen and Laine (16), following the Winkler (17) procedure in which tryptophane is allowed to react with glyoxylic acid in the presence of copper sulfate, reported 1.2 per cent in casein and Shaw and McFarlane (18) found 1.19 per cent colorimetrically and less than 1 per cent by isolation. Holiday (19) by a spectrophotometric method calculated that casein contains about 1 per cent tryptophane.

There has thus for years been a wide·divergence of findings as to the tryptophane content of casein, with values ranging from 0.51 to 2.4 per cent. The cause of the variation probably lies in the high reactivity of tryptophane—its sensitivity to excess of reactants and the oxidizing agents which have been used, the ease of destruction of tryptophane blue by oxidation, the different rates of color development of free tryptophane and tryptophane in casein, and the varying times at which color comparison has been made. Because of our earlier findings with the Bates procedure (4), and of the results of Virtanen and Laine (16), and of Shaw and McFarlane (18) who, with glyoxylic acid as reactant and copper sulfate as oxidant, obtained only 1.2 per cent tryptophane, we have reinvestigated the question of determining tryptophane not only in casein but also in other proteins.

In our early work (3) and in the corroborating work of Milone and Everitt (20) with a number of proteins, attention was called to the fact that by treating protein with *p*-dimethylaminobenzaldehyde in 20 per cent hydrochloric acid, warming, and oxidizing with dilute hydrogen peroxide, the same value was obtained as by the long May and Rose (1) procedure carried out at 37° without the use of hydrogen peroxide. The short method we regarded as a time saver, but considered also that the Bates procedure was preferable, since the reactions were carried out at room temperature. By modifying the Bates procedure so that dilution to volume was made with 10 to 20 per cent hydrochloric acid, tryptophane could be used as a standard. A further improvement was the use of the Klett-Summerson photoelectric colorimeter instead of the visual colorimeter. With this instrument, changes in the color could be readily followed over a period of time. The casein employed was made according to the directions of Van Slyke and Baker (21).

With the use of the May and Rose procedure with the Klett-Summerson

colorimeter, Filter 54, it was found that tryptophane gave a maximum blue color within 3 days, which then decreased to a plateau that was stable from the 6th to the 7th day. Casein on the other hand slowly increased in color and reached a maximum in 5 days, which stayed practically constant for 4 more days. If the maximum reading of the casein color was compared with the low relatively stable reading of the tryptophane color, the tryptophane value of casein could be estimated to be from 2 to 2.21 per cent, corrected for moisture and ash. However, if the maximum for the tryptophane and the maximum for the casein were taken as comparison points, the tryptophane content of casein was found to be 1.37 per cent. This value is too high, since the first readings were made after 3 days standing, when the readings for the tryptophane were going down and those for the casein were still increasing.

This procedure is essentially that of May and Rose as modified by Holm and Greenbank and used by Sullivan, Milone, and Everitt. By use of the photoelectric colorimeter, it became evident that the tryptophane color is slowly destroyed by oxidation in air, while the tryptophane color in the casein is not so destroyed.

Several experiments were made with use of the short procedure of Sullivan, Milone, and Everitt but at room temperature and with the use of 0.1 cc. of 0.3 per cent hydrogen peroxide, a 15 minute interval being allowed before the first reading. Depending on whether color comparison was made at the maximum readings in the tryptophane standard and in the casein solution or at a later relatively stable level, varying results were obtained. The comparison at the maximum readings gave a value of 1.77 per cent tryptophane in casein, the stable levels 2.5 per cent.

On the assumption that in this procedure the tryptophane had passed its maximum in the 15 minute wait before the first colorimetric reading, which was the highest, the experiment was repeated with a shorter period of waiting, the readings being begun as soon as the peroxide was added. In the case of free tryptophane, a reading of 315 was obtained within 3 minutes, which then fell off rapidly to a plateau. To come to a maximum plateau the casein required 3 to 6 hours, depending upon the room temperature. When the maximum readings in both cases were employed for the calculation, there was found for casein a value of 1.15 per cent tryptophane after correction for moisture and ash. To answer the question whether the tryptophane content of casein is 2.5 or 1.2 per cent other procedures were employed.

Bates (4) Procedure—To 50 mg. of casein in 2 cc. of 0.1 N sodium hydroxide were added 0.5 cc. of the *p*-dimethylaminobenzaldehyde reagent, 0.2 cc. (2 mg.) of $NaNO_3$ (the $NaNO_3$ contains a trace of $NaNO_2$), and 25 cc. of concentrated hydrochloric acid. After color development for 15 to 20

minutes, we modified the Bates procedure by diluting to 50 cc. with 17.5 per cent hydrochloric acid instead of with water, and read in the photoelectric colorimeter against tryptophane similarly treated. The average of seven determinations by the Bates short procedure as thus modified was 1.17 per cent tryptophane in casein, corrected for moisture and ash.

Shaw and McFarlane (18) Procedure—The Shaw and McFarlane procedure was slightly modified as follows: To 1 cc. of a 0.5 per cent solution of casein in 1.0 N sodium hydroxide and to the standard tryptophane solution, of which 0.5 cc. contained 0.05 mg., in an ice bath were added 0.5 cc. of freshly made glyoxylic acid solution (prepared according to Shaw and McFarlane (18)) and 0.4 cc. of 0.4 M copper sulfate solution and water to 3 cc. The mixture was cooled in ice water and 5 cc. of concentrated sulfuric acid were added, 0.5 cc. at a time so that no rise in temperature occurred. After standing for 10 minutes in the ice bath, the mixtures were heated for 5 minutes in a boiling water bath, cooled, and diluted to 10 cc. with approximately 65 per cent sulfuric acid. The tryptophane found after deduction of a casein blank was 1.26 per cent, corrected for moisture and ash. With omission of the glyoxylic acid we found a blank reading for both tryptophane and casein. If the blank was deducted for tryptophane as well as for casein, the tryptophane content of casein became 1.5 per cent.

Folin-Ciocalteu (22) Procedure—Folin and Ciocalteu estimated tryptophane by the color produced by their phenol reagent and reported a tryptophane value of 1.4 per cent in casein. Using this procedure on our casein and using the photoelectric colorimeter, we obtained a value of 1.28 per cent tryptophane, corrected for moisture and ash.

The four different procedures thus gave approximately the same value for the tryptophane content of casein: (a) the color generated by the reaction of casein with p-dimethylaminobenzaldehyde and with hydrogen peroxide as accelerator, the maximum color values being compared, gave 1.2 per cent; (b) the Bates procedure with sodium nitrate as accelerator gave 1.2 per cent; (c) the Shaw and McFarlane, 1.26 per cent; (d) the Folin-Ciocalteu 1.28 per cent. Accordingly it would seem that the tryptophane content of casein is 1.2 to 1.3 per cent.

The higher values by the May and Rose procedure as employed by various investigators, who found 2 per cent and over of tryptophane in casein, are doubtless due to the fact that comparison of the color generated by free tryptophane as a standard and by the tryptophane of the casein was made after the tryptophane standard had passed the maximum of color development. When comparison is made of the maximum readings for free tryptophane and for casein, the result obtained is approximately 1.2 per cent tryptophane in casein.

Tryptophane Content of Various Proteins—Since there was substantial

agreement in the results of the estimation of tryptophane in casein by a number of different methods, a study was made of the tryptophane content of squash seed globulin prepared by the method of Vickery, Smith, Hubbell, and Nolan (23), of crystalline egg albumin prepared by the method of Kekwick and Cannan (24), and of pepsin prepared by the procedure of Philpot (25).

Two procedures were employed, the Bates short procedure and that of Folin and Ciocalteu. Both of these methods are good but that of Bates is preferred, being more rapid and simpler. These methods were applied (1) to the unhydrolyzed protein, (2) to the protein hydrolyzed for 18 to 24 hours with 20 per cent sodium hydroxide, and (3) to the proteins hydrolyzed for 4 hours with 20 per cent hydrochloric acid containing butyl alcohol.

TABLE I

Percentage Content of Tryptophane in Casein, Squash Seed Globulin, Crystalline Egg Albumin, and Crystalline Pepsin by Bates and Folin-Ciocalteu Procedures

Protein	Unhydrolyzed protein	Sodium hydroxide hydrolysate		Acid hydrolysate
	Bates	Bates	Folin-Ciocalteu	Bates
Casein	1.17	1.24	1.24	0.42
Squash seed globulin	1.69	1.71	1.71	1 41
Egg albumin	1 23	1.22	1.25	0.66
Pepsin	2.25	2 16	2 21	1.24

Alkali Hydrolysis—A mixture containing 100 to 500 mg. of the protein, 10 cc. of 20 per cent sodium hydroxide, and 1.0 cc. of butyl alcohol was heated for 18 hours at 125°, cooled, and acidified with 7.5 cc. of 14 N sulfuric acid, diluted to 50 cc. with water, and filtered. Aliquots, 5 cc. for the Bates procedure and 8 cc. for the Folin-Ciocalteu procedure, were employed.

Acid Hydrolysis—A mixture of 100 to 200 mg. of protein, 200 mg. of urea, 2 cc. of 20 per cent hydrochloric acid, and 1 cc. of butyl alcohol was heated for 4 hours at 125–130° with a stream of nitrogen passing through the solution. On be ng cooled, the solution was brought to pH 3.5 and made to 20 cc. with water. A 5 cc. aliquot was analyzed by the Bates short procedure.

The findings, based on two to six determinations in each case, are given in Table I. The values are corrected for moisture and ash.

In the acid-hydrolyzed protein, much less tryptophane was found than in that hydrolyzed by alkali or in the unhydrolyzed protein, possibly because of destruction by the acid of the bound tryptophane. Pure tryptophane similarly hydrolyzed for 4 hours suffers no apparent loss inasmuch as the same colorimetric reading is obtained both with and without acid treatment. However, practically the same tryptophane values are obtained from the

unhydrolyzed protein as from the protein hydrolyzed for at least 18 hours with 20 per cent sodium hydroxide with both the Bates and the Folin-Ciocalteu procedures.

SUMMARY

The great divergence among findings of the tryptophane content of casein, with values ranging from 0.51 to 2.4 per cent, is due mainly to the different rates of color development of free tryptophane and tryptophane combined in the protein. The values of 2 per cent and more result from comparison of the color generated by the standard and unknown at points other than those at which color formation is maximum.

By employing p-dimethylaminobenzaldehyde and using the maximum readings in the Klett-Summerson photoelectric colorimeter, Filter 54, the tryptophane content of casein was found to be between 1.2 and 1.3 per cent. These values were corroborated by four different procedures. Of these procedures that of Folin and Ciocalteu and a modification of that of Bates were found especially satisfactory, with preference given to the latter on grounds of simplicity. Both yield concordant values respectively for casein, squash seed globulin, and pepsin.

BIBLIOGRAPHY

1. May, C E , and Rose, E R , *J. Biol Chem* , **54,** 213 (1922)
2. Holm, G E , and Greenbank, G R , *J Am Chem Soc* , **45,** 1788 (1923).
3. Sullivan, M X , Milone, H S , and Everitt, E L , *J Biol. Chem* , **125,** 471 (1938).
4. Bates, R W., *Proc Am Soc Biol Chem* , *J Biol Chem.*, **119,** p vii (1937)
5. Furth, O , and Nobel, E , *Biochem Z* , **109,** 103 (1920)
6. Furth, O , and Lieben, F , *Biochem. Z* , **109,** 124 (1920).
7. Thomas, P , *Ann Inst Pasteur,* **34,** 701 (1920)
8. Komm, E , and Bohringer, E , *Z physiol. Chem* , **124,** 287 (1923).
9. Jones, D B., Gersdorff, C E F , and Moeller, O., *J. Biol Chem* , **62,** 183 (1924–25).
10. Hopkins, F G , and Cole, S W , *J Physiol* , **27,** 418 (1901–02)
11. Dakin, H. D , *Biochem J* , **12,** 290 (1918)
12. Fasal, H , *Biochem Z* , **44,** 392 (1912).
13. Herzfeld, E , *Biochem Z* , **56,** 258 (1913).
14. Homer, A , *J. Biol Chem* , **22,** 369 (1915)
15. Lüscher, E , *Biochem J.*, **16,** 556 (1922).
16 Virtanen, A I , and Laine, T , *Biochem J* , **30,** 1509 (1936)
17. Winkler. S , *Z physiol Chem* , **228,** 50 (1934).
18. Shaw, J L D., and McFarlane, W D , *Canad. J Res* , *Sect B,* **16,** 361 (1938).
19. Holiday, E R , *Biochem J* , **30,** 1795, 1799 (1936)
20. Milone, H S , and Everitt, E L., *Proc Soc Exp Biol. and Med.*, **51,** 82 (1942).
21. Van Slyke, L L., and Baker, J C., *J. Biol Chem.*, **35,** 127 (1918)
22. Folin, O , and Ciocalteu, V , *J. Biol Chem.*, **73,** 627 (1927)
23. Vickery, H B., Smith, E L , Hubbell, R. B., and Nolan, L S , *J. Biol Chem* , **140,** 613 (1941).
24. Kekwick, R A., and Cannan, R K , *Biochem J* , **30,** 227 (1936)
25. Philpot, J. S., *Biochem J* , **29,** 2458 (1935)

NATURALLY OCCURRING GLYCEROL ETHERS

II. SYNTHESIS OF SELACHYL ALCOHOL*

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The occurrence of at least three α-glycerol ethers, batyl alcohol, chimyl alcohol, and selachyl alcohol, in the unsaponifiable matter of oils from various marine animals, especially fish liver oils, has been well established (2). Recently batyl alcohol has been isolated also from other sources, which makes it appear that glycerol ethers occur much more widely in the animal organism than was originally suspected. Kind and Bergmann (3) obtained the alcohol from the unsaponifiable matter of the reef-building gorgonia, *Plexaura flexuosa.* Bergmann and Stansbury (4) demonstrated that "astrol," isolated by Kossel and Edlbacher as early as 1915 (5) from the starfish, *Asterias rubens*, is identical with batyl alcohol. The isolation of batyl alcohol from the yellow bone marrow of cattle by Holmes, Corbet, Geiger, Kornblum, and Alexander (6), from the spleen of pigs by Prelog, Ruzicka, and Stein (7), and from arteriosclerotic arteries of human beings by Hardegger, Ruzicka, and Tagmann (8) proved the occurrence of this type of substance also in higher animals.

Both enantiomorphs of batyl alcohol and chimyl alcohol were synthesized in this Laboratory several years ago (1) by condensation of the sodium salts of $d(+)$-acetone glycerol and $l(-)$-acetone glycerol with n-octadecyl iodide and n-hexadecyl iodide and subsequent removal of the protecting acetone group by acid hydrolysis. d-α-(n-Octadecyl) glycerol and d-α-(n-hexadecyl) glycerol prepared from $l(-)$-acetone glycerol were found to be identical with natural batyl alcohol and chimyl alcohol respectively, thus proving for both natural products the d configuration. On catalytic reduction of selachyl alcohol d-batyl alcohol was obtained (9). Selachyl alcohol, therefore, also belongs to the d series. The steric relationship of these ethers was assigned according to the principle used for the classification of α-monoglycerides (10) and glycerophosphates.

Although the constitution and the configuration of the natural selachyl alcohol were proved beyond doubt, we felt that its synthesis was desirable, since, in analogy to the wide-spread and plentiful occurrence of-oleic acid in nature, it seems to occur much more widely than any of the other glycerol

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* First communication, "Configuration of the natural, batyl, chimyl, and selachyl alcohols," (1).

ethers. The synthesis was carried out on lines similar to that of batyl and chimyl alcohol (1) except that we preferred to use oleyl-p-toluenesulfonate instead of the halogen derivatives of oleyl alcohol. The p-toluenesulfonate was chosen because it can be readily prepared without affecting the ethylenic linkage, reacts more readily than the corresponding halogen derivatives (11), and any unchanged material is easily separated. The oleyl-p-toluenesulfonate was prepared in the usual way (11) from oleyl alcohol which had been carefully purified by low temperature crystallization from acetone and fractional distillation in a high vacuum, since the success of the synthesis depends on the use of pure oleyl alcohol. The oleyl-p-toluenesulfonate reacted readily with the sodium salts of $d(+)$-, $l(-)$-, and dl-acetone glycerol (A) in boiling glycol dimethyl ether, forming the acetone compounds of the three selachyl alcohols. These compounds were isolated in pure form in yields of from 64 to 67 per cent. Removal of the protecting acetone group by hydrolysis with 80 per cent acetic acid at 80° (B) yielded the pure d- and l-selachyl alcohol, m.p. 48.5–49.5°, and dl-selachyl alcohol, m.p. 46.5–47.5°.

$$\text{CH}_2\text{ONa}$$

$$
\begin{array}{c}
\text{H}_3\text{C} \quad\quad \text{OCH} \\
\diagdown \;\; \diagup \quad\;\; | \\
\text{C} \quad\quad | \\
\diagup \;\; \diagdown \quad\;\; | \\
\text{H}_3\text{C} \quad\quad \text{OCH}_2
\end{array}
\quad + \; \text{C}_{18}\text{H}_{35}\text{O}\cdot\text{SO}_2\cdot\text{C}_7\text{H}_7 \;\xrightarrow{\text{(A)}}
$$

Sodium salt of $l(-)$-acetone glycerol

$$\text{CH}_2\cdot\text{O}\cdot\text{C}_{18}\text{H}_{35}$$

$$
\begin{array}{c}
\text{H}_3\text{C} \quad\quad \text{OCH} \\
\diagdown \;\; \diagup \quad\;\; | \\
\text{C} \quad\quad | \\
\diagup \;\; \diagdown \quad\;\; | \\
\text{H}_3\text{C} \quad\quad \text{OCH}_2
\end{array}
\quad + \; \text{NaOSO}_2\cdot\text{C}_7\text{H}_7 \;\xrightarrow{\text{(B)}}\;
\text{HO}\!-\!\underset{\underset{\text{H}_2\text{COH}}{|}}{\overset{\overset{\text{CH}_2\cdot\text{O}\cdot\text{C}_{18}\text{H}_{35}*}{|}}{\text{C}}}\!-\!\text{H}
$$
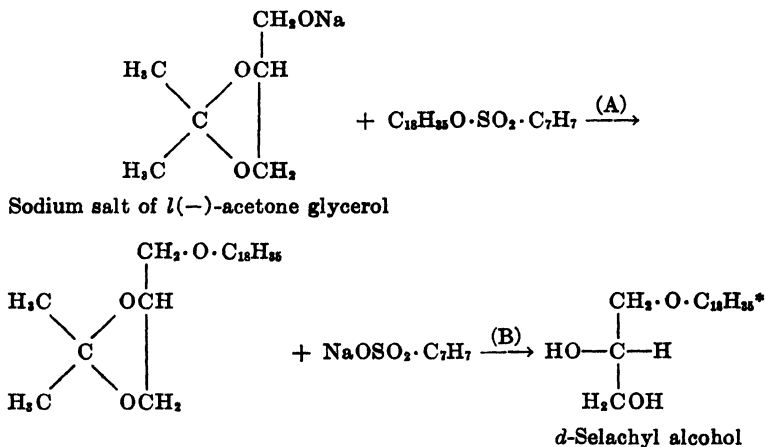
d-Selachyl alcohol

The good agreement of the rotations of the natural selachyl alcohol ($[\alpha]_D^{15} = -4.5°$, in substance, Toyama (9)) and its acetate ($[\alpha]_D^{15} = -8.6°$, in substance (9)) with the rotations of the synthetic selachyl alcohol ($[\alpha]_D^{55} = -4.35°$) and its acetate ($[\alpha]_D^{23} = -8.6°$) derived from $l(-)$-acetone glycerol confirms our earlier inference (1) that the natural selachyl alcohol also belongs to the d series.

Selachyl alcohol has been isolated from natural sources so far only as an oil. The fact that the alcohol is accompanied in nature by both saturated

* Please note that in the paper by Baer and Fischer ((1) p. 399, below the formula) R should read for selachyl alcohol $C_{18}H_{35}$ and for batyl alcohol $C_{18}H_{37}$.

and highly unsaturated related compounds might be responsible for the failure to obtain it in the crystalline state. Our synthetic selachyl alcohols prepared from highly purified oleyl alcohol crystallized readily. To rule out the possibility of elaidinization oleyl alcohol was subjected to the conditions of the synthesis. It was found that oleyl alcohol remained essentially unchanged. This, along with the fact that sodium alcoholates do not bring about elaidization (12), assured us that no *cis-trans* isomerization occurred during the synthesis and that our synthetic products are pure *α-oleyl* glycerols.

Beyond the fact that the α-glycerol ethers have been isolated from the most diverse sources nothing is known so far about their biological significance or how they are formed in metabolism. It is conceivable that these ethers are formed from phosphatidic acids (I) in the following way: In the first step these acids rearrange to form a "hemiorthoester" (II). This type of compound was already suggested by E. Fischer, Bergmann, and Lipschitz (13) to play a rôle in the acyl migration of depsides and glycerides and has been synthesized later by Hibbert and Greig (14). The reduction of (II) leads to plasmals (III) whose existence has been discovered by Feulgen, Imhäuser, Behrens, and Grünberg (15). The reduction of plasmals and subsequent removal of phosphoric acid yield (IV) and (V).

The fact that natural α-glycerophosphoric acid belongs to the *l* series (16), and that the glycerol ethers, as far as they have been investigated, belong to the *d* series (1), is in agreement with the hypothesis and prompts us to propose this tentative scheme for the formation of α-glycerol ethers in nature.

$$
\begin{array}{ccc}
\mathrm{R-C-O-CH_2} & & \mathrm{R-C-O-CH_2} \\
\parallel \quad \mathrm{\ \ |} & & \diagup \ \ \diagdown \quad | \\
\mathrm{O \quad HOC-H} & \longrightarrow & \mathrm{HO \qquad O-CH} \\
\mathrm{\quad \ \ |} & & \mathrm{\qquad \quad |} \\
\mathrm{CH_2-OPO_3H_2} & & \mathrm{CH_2-OPO_3H_2} \\
\text{(I)} & & \text{(II)}
\end{array} \xrightarrow{\ \mathrm{H_2}\ }
$$

$$
\begin{array}{ccc}
\mathrm{R-C-O-CH_2} & & \mathrm{R-CH_2-O-CH_2} \\
\diagup \ \ \diagdown \quad | & & | \\
\mathrm{H \qquad O-CH} & \xrightarrow{\mathrm{H_2}} & \mathrm{HOC-H} \\
\mathrm{\qquad \quad |} & & | \\
\mathrm{CH_2-OPO_3H_2} & & \mathrm{CH_2-OPO_3H_2} \\
\text{(III)} & & \text{(IV)}
\end{array} \longrightarrow
$$

$$
\begin{array}{c}
\mathrm{R-CH_2-O-CH_2} \\
| \\
\mathrm{HO-C-H} \\
| \\
\mathrm{CH_2OH} \\
\text{(V)}
\end{array}
$$
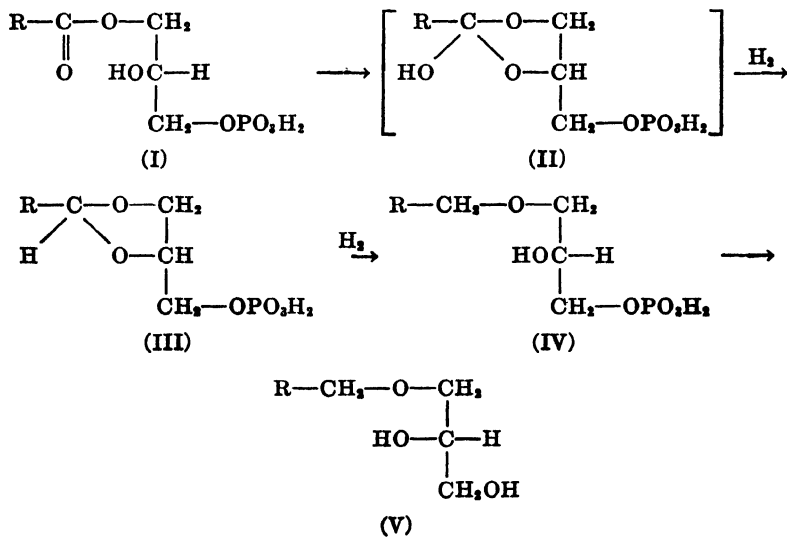
Purification of Oleyl Alcohol—Oleyl alcohol was prepared by high pressure reduction of methyl oleate. The crude oleyl alcohol (iodine number 83.5) contained approximately 16 to 17 per cent of saturated alcohols (mainly cetyl alcohol) and about 6 per cent of more highly unsaturated alcohol (linoleyl alcohol). The accompanying substances were removed by low temperature crystallization from acetone according to the procedure for the purification of oleic acid developed by Brown and Shinowara (17) and Wheeler and Riemenschneider (18). The crystallizations and filtrations were carried out in a straight walled separatory funnel which was surrounded by a bath filled with a freezing mixture of suitable tempera-

TABLE I

Progress of Purification of Oleyl Alcohol

Fraction No	Weight	Iodine No., Wijs, 30 min.	n_D^{22}
	gm.		
1. Cetyl alcohol	9.0	26.1	
2. Residue* from 2nd mother liquor	8.0	123.0	1.4703
3. " " 3rd " "	2.4	105.7	1.4639
4. " " 4th " "	1.24	96.5	1.4610
5. " " 5th " "	0.95	91.8	1.4600
6. Oleyl alcohol fraction	27.1	87.3	1.4592

* Fractions 2 to 5 were obtained on evaporation of the mother liquors of the four recrystallizations at −60°.

ture. The funnel contained a filter plug of glass wool held down by a perforated porcelain plate wrapped in filter paper.

A solution of 50 gm. of crude oleyl alcohol in 475 cc. of acetone was transferred to the low temperature crystallization apparatus and kept at −20° to −25°. Cetyl alcohol crystallized out. After standing for 1.5 hours at this temperature, with occasional stirring, the mixture was filtered with suction and the cetyl alcohol washed three times with 20 cc. portions of cold acetone (−25°). The filtrate and washings were made up to 500 cc., if necessary, transferred to the crystallization apparatus, cooled to −60° with frequent stirring, and kept at this temperature for 1 hour. The mother liquor was sucked off and the oleyl alcohol was washed on the filter three times with cold acetone (−60°). The filtrate and washings contained the more highly unsaturated alcoholic fraction which was recovered and analyzed. The oleyl alcohol was washed through the filter with warm acetone and the solution made up to 500 cc. The crystallization from acetone at −60° was repeated three times. Table I gives the

weight, iodine number, and refractive index of the material removed during the first part of the purification of the crude oleyl alcohol.

From the data presented it is evident that the oleyl alcohol (Fraction 6) has been freed from the linoleyl alcohol but still contains approximately 8 per cent of cetyl alcohol which could not be removed by crystallization.

For further purification 55 gm. of Fraction 6 were fractionally distilled through a 55 cm. column of 17.5 mm. internal diameter, packed with glass cylinders (6 × 6 mm). The column which was surrounded by a well insulated jacket was heated electrically over its entire length. The head was of the total condensation variable take-off type. To prevent excessive bumping glass wool was used.[1]

The temperature of the jacket and the oil bath was so adjusted that the bottom of the packing was slightly flooded. 20 per cent of the weight of the starting material, approximately 2.5 times the amount of cetyl alcohol calculated to be present, was taken as precursor (b.p. 143°, 0.9 mm.). The first portion of the precursor contained so much cetyl alcohol that in order to prevent its solidification the still head had to be slightly warmed. The oleyl alcohol fraction which came over next boiled in a vacuum of 0.5 mm. at 145–146°. The oleyl alcohol was a colorless and odorless oil. Yield 25 gm.; $n_D^{27} = 1.4582$, $n_D^{20} = 1.4606$, $n_D^{15} = 1.4622$; $d^{26} = 0.842$; m.p. 5.5–7.5°. Iodine number (semimicro, Hanus, 30 minutes), calculated 94.5, found 94.1. The purity of the oleyl alcohol thus prepared is 99.7 per cent, and is greater than any reported so far in the literature (19).

Oleyl-p-toluenesulfonate—To a solution of 26.8 gm. (0.1 mole) of oleyl alcohol in 32 cc. of dry pyridine were added with stirring over a period of 30 minutes 20.9 gm. (0.11 mole) of pure p-toluenesulfonyl chloride and the stirring was continued for at least 3 hours, the temperature being maintained between 10–20°. To destroy the excess of acid chloride 1 cc. of water was added and the mixture was allowed to stand overnight. 250 cc. of ice-cold 2.5 N HCl were added and the oleyl-p-toluenesulfonate was separated by gently swirling[2] the aqueous layer with several portions of peroxide-free ether. The combined ether extracts were washed twice with a saturated sodium chloride solution, saturated sodium bicarbonate solution, and water, and dried with sodium sulfate. When the ether was distilled off and the last traces of the solvent removed *in vacuo* at 100°, 38.3 gm. (90.5 per cent) of crude oleyl-p-toluenesulfonate were obtained. The ester was recrystallized from 50 cc. of petroleum ether (b.p. 40–60°) at −30°, yielding 26.6 gm. (70 per cent) of pure p-toluenesulfonate. The sub-

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[1] The glass joint was lubricated with a water suspension of aquadag (colloidal graphite).

[2] In order to avoid the formation of stable emulsions.

stance was obtained in fine, white crystals with a melting point of 18.5–
19.5°; $n_D^{26.5} = 1.4885$. The ester is not distillable.

$C_{25}H_{42}O_3S$ (422.7). Calculated, S 7.58; found 7.53, 7.60

Oleyl Pyridinium-p-toluenesulfonate—A mixture of 5.0 gm. of oleyl-*p*-
toluenesulfonate and 1.05 gm. of dry pyridine was kept at 130° for 2 hours.
The solution of the semisolid reaction mass in 15 cc. of boiling acetone
yielded on cooling 5.0 gm. (84.2 per cent) of the pyridinium compound,
which after repeated crystallization from acetone melted at 123–124.5°
(corrected).

$C_{30}H_{47}O_3SN$ (501.8). Calculated, S 6.39, N 2.79; found, S 6.46, N 2.79

Oleyl-β-naphthylurethane[3]—A solution of 0.51 gm. of oleyl alcohol and
0.32 gm. of β-naphthyl isocyanate[4] in 5 cc. of dry benzene was refluxed for
2 hours. The solution was freed from dinaphthylurea and evaporated to
dryness *in vacuo*. The residue (0.69 gm., 83.5 per cent) was crystallized
five times from ethanol to a constant melting point. M.p. 46–47°. (André
and François (20) reported 44–45°.)

$C_{29}H_{43}O_2N$ (437.6). Calculated, N 3.20; found, 3.16

d(+)- and l(−)-Acetone Glycerol—Both substances were prepared ac-
cording to the simplified procedure reported by us for the preparation of
d(+)-acetone glycerol (21); however, we used for the reduction of the ace-
tone glyceraldehydes the more readily obtainable Raney's nickel instead
of Rupe's nickel catalyst. The specific rotation of both acetone glycerols
was in general between ±14.6° and ±14.8°.

Acetone Compound of d-Selachyl Alcohol—The preparation of the sodium
salt of *l*(−)-acetone glycerol was carried out in a 250 cc. flask with standard
tapers in an atmosphere of pure nitrogen.

50.0 cc. of a molar solution of sodium naphthalene in glycol dimethyl
ether were prepared according to the directions of Scott, Walker, and
Hansley (22). Into this solution, cooled with water, was run with stirring
a solution of 7.26 gm. of freshly prepared *l*(−)-acetone glycerol in 15 cc.
of dry glycol dimethyl ether and the stirring continued until the formation
of the sodium compound of acetone glycerol was completed, which was
indicated by the disappearance of the green color. A solution of 21.1 gm.
of oleyl-*p*-toluenesulfonate in 50 cc. of glycol dimethyl ether was added and
the mixture was gently refluxed for 24 hours. The sodium *p*-toluenesul-

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[3] This compound was reported by André and François (20) without any experi-
mental details being given.

[4] The commercially available β-naphthyl isocyanate contains various amounts
of symmetrical dinaphthylurea. A separation of both compounds is easily effected
with anhydrous ether.

fonate was removed, the solvent was distilled off, and the residue was gradually heated to a temperature of 160° (bath) in a vacuum of 10 to 15 mm. in order to remove most of the naphthalene and dihydronaphthalene. The remaining light brown oil was taken up in ether. The solution was washed three times with water, dried over sodium sulfate, and again concentrated. For further purification of the crude material, which still contained naphthalene, dihydronaphthalene, and oleyl-p-toluenesulfonate, it was found advantageous to distil the mixture first in a molecular still (23). The distillation was carried out at a speed of 1 drop per 15 to 20 seconds in a vacuum of 1×10^{-3} mm. and at 85-90° (air bath). The product, now free from the non-distillable sulfonate, but still containing traces of both naphthalenes, was fractionally distilled *in vacuo*, in an all-glass sword flask with a sealed-on receiver.[5] Pure nitrogen was introduced through the capillary. A small precursor was taken which contained the impurities. The pure acetone compound of d-selachyl alcohol distilled in a vacuum of 1×10^{-3} mm. at 152-154° (oil bath 185-195°). Yield 12.9 gm. (67.5 per cent); $n_D^{25.5} = 1.4570$; $d^{24.5} = 0.891$.

$C_{24}H_{48}O_3$ (382.6). Calculated. C 75.3, H 12.13, acetone 15.18
Found.   " 75.4, " 12.15,   "   14.81
Optical Rotation—In substance, 1 dm. tube, $\alpha_D^{24.5} = -11.52°$, $[\alpha]_D^{24.5} = -12.9°$.

Acetone Compound of l-Selachyl Alcohol—The condensation of 7.2 gm. of $d(+)$-acetone glycerol with 21.1 gm. of oleyl-p-toluenesulfonate carried out as described for the isomer yielded 12.4 gm. (64.6 per cent) of the acetone compound of l-selachyl alcohol, b.p. $(6 \times 10^{-3}$ mm.) = 160-165° (bath, 180-190°). $n_D^{21.5} = 1.4570$; $d^{22} = 0.895$.

Calculated, acetone 15.18; found, 14.62
Optical Rotation—In substance, 1 dm. tube, $\alpha_D^{22} = +11.46°$, $[\alpha]_D^{22} = +12.8°$.

Acetone Compound of dl-Selachyl Alcohol—7.2 gm. of racemic acetone glycerol on condensation with 21.1 gm. of oleyl-p-toluenesulfonate yielded 12.7 gm. (66.3 per cent) of the acetone compound of racemic dl-selachyl alcohol, b.p. $(4 \times 10^{-3}$ mm.) = 160-163° (bath, 200-207°); $n_D^{24.5} = 1.4560$.

Calculated. C 75.3, H 12.13, acetone 15.18
Found.   " 75.2, " 11.98,   "   14.68

d-Selachyl Alcohol—15.0 gm. of the acetone compound of d-selachyl alcohol were dissolved in 125 cc. of 80 per cent acetic acid at 80° and the solution was kept at this temperature for 2 hours. On the addition of 600 cc.

[5] This distilling flask has been described ((24), Fig. 2). All distillations reported in this communication were carried out in sword flasks unless otherwise specified. These details are mentioned because the boiling points in high vacuum are largely dependent on the type of flask used.

of cold water the selachyl alcohol precipitated as a semisolid mass which on cooling hardened and could be filtered. In order to saponify any acetic acid ester formed during the deacetonation process, the substance was triturated with an excess of dilute potassium hydroxide solution on a steam bath for 30 minutes. The product was taken up in ether, the ether washed with water until free from alkali, and the solution dried over sodium sulfate. On removal of the ether 12.7 gm. (94.7 per cent) of crude d-selachyl alcohol were obtained. Recrystallization from 30 cc. of acetone at $-10°$ yielded 7.3 gm. of pure selachyl alcohol. M.p. 48–49°. Two more crystallizations from acetone raised the melting point to 48.5–49.5°. $d^{55} = 0.888$.

$C_{21}H_{42}O_3$ (342.6). Calculated. C 73.6, H 12.32, I No. 74.0
Found.   " 73.3, " 12.48, " " 72.9 (Hanus, 30 min.)

l-Selachyl Alcohol—The acid hydrolysis of 1.6 gm. of the acetone compound of *l*-selachyl alcohol yielded 1.26 gm. (88 per cent) of *l*-selachyl alcohol. Repeated crystallization (six times) from acetone and once from methanol raised the melting point to 48.5–49.5°.

Calculated. C 73.6, H 12.32, I No. 74.0
Found.   " 73.7, " 12.00, " " 72.9

dl-Selachyl Alcohol—The acid hydrolysis of 10.6 gm. of the acetone compound of *dl*-selachyl alcohol in 80 per cent acetic acid at 80° yielded 9.0 gm. (94.7 per cent) of *dl*-selachyl alcohol. Repeated crystallization from acetone and once from methanol gave pure *dl*-selachyl alcohol, m.p. 46.5–47.5°.

Calculated. C 73.6, H 12.32, I No. 74.0
Found   " 73.6, " 12.27, " " 73.0

Optical Rotation—(a) Synthetic d-selachyl alcohol in melted substance, 1 dm tube, $\alpha_D^{55} = -3.86°$, $[\alpha]_D^{55} = -4\ 35°$ Toyama (9) reports for the natural selachyl alcohol $[\alpha]_D^{14} = -4.5°$.

(b) The rotations for the natural and synthetic product in solvents are shown in Tables II and III.

Diacetyl d-Selachyl Alcohol—1.5 gm. of d-selachyl alcohol were dissolved in a mixture of 2.4 cc. of acetic anhydride and 4.3 cc. of dry pyridine. After standing for 24 hours at room temperature the solution was concentrated *in vacuo* and the residue distilled under a high vacuum in a flask with a sealed-on receiver. B.p. (1×10^{-3} mm.) = 170–172° (oil bath, 210–220°). Yield 1.77 gm. (94.7 per cent) of diacetyl selachyl alcohol. M.p. 18–19°; $n_D^{24.5} = 1.4521$; $d^{23} = 0.937$.

$C_{25}H_{46}O_5$ (426.6). Calculated, C 70.4, H 10.87; found, C 70.3, H 10.50
Optical Rotation—In substance, 1 dm. tube, $\alpha_D^{23} = -8.07°$, $[\alpha]_D^{23} = -8.6°$ (Toyama (9) reports $[\alpha]_D^{?} = -8.6°$). In dry ethanol, 2 dm. tube, $c = 10.44$, $\alpha_D^{24} = -1.88°$, $[\alpha]_D^{24} = -9.0°$ (Toyama and Ishikawa (25) report $[\alpha]_D^{23} = -8.64°$).

Diacetyl l-Selachyl Alcohol—The diacetate was prepared from *l*-selachyl alcohol as described above.   Yield 90 per cent; b.p. (1 × 10⁻² mm.) = 182–183° (oil bath, 220°); $n_D^{23.5}$ = 1.4530; d^{23} = 0.935.

TABLE II

Optical Rotation in Dry Ethanol and in Ethanol-Free Chloroform *

l-Selachyl alcohol, synthetic product		*d*-Selachyl alcohol, synthetic product		*d*-Selachyl alcohol, natural product†	
Concentration	$[\alpha]_D$	Concentration	$[\alpha]_D$	Concentration	$[\alpha]_D$
Dry ethanol					
	degrees		*degrees*		*degrees*
20.4	+0.6	20.6	−0.6	20.0	−0.6
10.0	0.0	10.0	0.0	10.0	0.0
5.0	−0.3	5.0	+0.2	5.0	+0.3
Ethanol-free chloroform					
23.3	+1.5	23.0	−1.3	23.0	−1.5
10.0	−0.9	10.0	+1.1	10.0	0.0
4.9	−2.2	5.1	+2.0	5.2	+0.8

* The rotations in ethanol-free chloroform differed considerably from the rotations in U. S. P. chloroform. This difference is apparently caused by the small amount of ethanol in U. S. P. chloroform.

† Toyama and Ishikawa (25).

TABLE III

*Optical Rotation of d-Selachyl Alcohol in U. S. P. Chloroform (Containing 1 Per Cent Ethanol)**

Synthetic product		Natural product (Toyama and Ishikawa (25))	
Concentration	$[\alpha]_D$	Concentration	$[\alpha]_D$
	degrees		*degrees*
20.1	−0.9	20.0	−1.1
12.2	+0.1	12.0	−0.2
10.1	+0.3	10.0	0.0
5.1	+1.2	5.0	+0.9

* See foot-note to Table II.

Calculated, C 70.4, H 10.87; found, C 70.0, H 10.50

Optical Rotation—In substance, 1 dm. tube, α_D^{23} = +8.11°, $[\alpha]_D^{23}$ = +8.7°.   In dry ethanol, 2 dm. tube, c = 10.53, α_D^{23} = +1.86°, $[\alpha]_D^{23}$ = +8.8°.

Diacetyl dl-Selachyl Alcohol—The acetylation of *dl*-selachyl alcohol was carried out as described above.   Yield 90 per cent; b.p. (5 × 10⁻³ mm.) = 175–176° (oil bath, 216–220°); $n_D^{25.5}$ = 1.4520.

Calculated, C 70.4, H 10.87; found, C 70.6, H 10.79

Diphenylurethane of d-Selachyl Alcohol—A mixture of 0.5 gm. of *d*-selachyl alcohol and 0.5 gm. of freshly distilled phenyl isocyanate was kept at 110° for half an hour. The excess of isocyanate was removed *in vacuo* at 100°. The solid residue was recrystallized from 5 cc. of methanol and yielded 0.63 gm. (74.4 per cent) of pure diphenylurethane, m.p. 93–94°.

$C_{38}H_{52}O_5N_2$ (580.8). Calculated, N 4.83; found, 4.82

Optical Rotation—In dry pyridine, 2 dm. tube, $c = 6.40$, $\alpha_D^{24.5} = -0\,77°$, $[\alpha]_D^{24.5} = -6.0°$.

Diphenylurethane of l-Selachyl Alcohol—0.5 gm. of *l*-selachyl alcohol yielded 0.59 gm. (69.5 per cent) of diphenylurethane, m.p. 93–94°.

Calculated, N 4.83; found, 5.0

Optical Rotation—In dry pyridine, 2 dm. tube, $c = 6.67$, $\alpha_D^{22} = +0.77°$, $[\alpha]_D^{22} = +5.8°$.

Diphenylurethane of dl-Selachyl Alcohol—0.5 gm. of *dl*-selachyl alcohol yielded 0.71 gm. (83.7 per cent) of diphenylurethane, m.p. 92–93°.

Calculated, N 4.83, found, 4.80

Attempt to Elaidinize Oleyl Alcohol under Conditions of the Synthesis—The sodium compound of 1.75 gm. of acetone glycerol was prepared as described above. 2.3 gm. of sodium *p*-toluenesulfonate and 1.5 gm. of pure oleyl alcohol were added and the mixture refluxed for 24 hours. To decompose the sodium alcoholates 2.0 cc. of 5.0 N sulfuric acid were added. The mixture was filtered and the filtrate worked up as in the case of the acetone compound of *d*-selachyl alcohol. 1.7 gm. of crude oleyl alcohol containing some naphthalene were recovered. A fractional distillation yielded 1.0 gm. of fairly pure oleyl alcohol, b.p. (1 mm.) 165° (bath temperature 190°); m.p. 3.5–6.5° (m.p. of pure oleyl alcohol 5.5–7.5°). For further identification of the oleyl alcohol its β-naphthylurethane was prepared. Yield 76 per cent; m.p. 44–46° (after two crystallizations from ethanol). Elaidyl alcohol, m.p. 36–37°; elaidyl-β-naphthylurethane, m.p. 71° (20).

SUMMARY

1. *d*-, *l*-, and racemic selachyl alcohol have been synthesized by condensation of oleyl-*p*-toluenesulfonate with the sodium salts of $l(-)$-, $d(+)$-, and racemic acetone glycerol and subsequent acid hydrolysis of the acetone compounds.

2. The comparison of the optical rotations of the synthetic selachyl alcohols and their acetyl derivatives with those of the natural selachyl alcohol and its acetate confirmed our earlier inference that the natural selachyl

alcohol, together with batyl alcohol and chimyl alcohol, belongs to the *d* series.

3. The synthetic selachyl alcohols are crystalline solids; *d*- and *l*-selachyl alcohols melt at 48.5–49.5°; the *dl*-selachyl alcohol melts at 46.5–47.5°. The natural selachyl alcohol up to now has been isolated only as an oil.

BIBLIOGRAPHY

1. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **140,** 397 (1941).
2. Hilditch, T. P., The chemical constitution of natural fats, London, 362 (1940). Schmidt-Nielsen, S., and Ârtun, T., *Kl. Norske Videnskb. Selskabs, Skrifter,* 45 (1940); *Chem. Abstr.*, **37,** 3510 (1943).
3. Kind, C. A., and Bergmann, W., *J. Org. Chem.*, **7,** 424 (1942).
4. Bergmann, W., and Stansbury, H. A., *J. Org. Chem.*, **8,** 283 (1943).
5. Kossel, A., and Edlbacher, S., *Z. physiol. Chem.*, **94,** 277 (1915).
6. Holmes, H. N., Corbet, R. E., Geiger, W. B., Kornblum, N., and Alexander, W., *J. Am. Chem. Soc.*, **63,** 2607 (1941).
7. Prelog, V., Ruzicka, L., and Stein, P., *Helv chim. acta*, **26,** 2222 (1943).
8. Hardegger, E., Ruzicka, L., and Tagmann, E., *Helv. chim. acta*, **26,** 2205 (1943).
9. Toyama, Y., *Chem. Umschau Fette, Öle, Wachse u. Harze*, **31,** 61 (1924).
10. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128,** 475 (1939).
11. Sereka, V. C , and Marvel, C. S., *J. Am. Chem. Soc.*, **55,** 345 (1933).
12. Bouveault, L., and Blanc, G., *Bull. Soc. chim.*, **31,** 1210 (1904).
13. Fischer, E., Bergmann, M , and Lipschitz, W., *Ber. chem. Ges.*, **51,** 45 (1918). Fischer, E., *Ber chem. Ges.*, **53,** 1621 (1920).
14. Hibbert, H., and Greig, M. E., *Canad. J. Res* , **4,** 254 (1931).
15. Feulgen, R , Imhàuser, K., and Behrens, M., *Z. physiol. Chem.*, **180,** 161 (1929). Feulgen, R., and Behrens, M., *Z. physiol. Chem.*, **255,** 15 (1938). Feulgen, R., and Grünberg, H., *Z. physiol. Chem.*, **257,** 161 (1939).
16. Baer, E., and Fischer, H. O L., *J. Biol. Chem.*, **128,** 491 (1939).
17. Brown, J. B., and Shinowara, G. Y., *J. Am. Chem. Soc.*, **59,** 6 (1937).
18. Wheeler, D. H., and Riemenschneider, R. W., *Oil and Soap*, **16,** 207 (1939).
19. Beilsteins Handbuch der organischen Chemie, Berlin, 4th edition, **1,** 453 (1918); 2nd suppl., **1,** 503 (1941). Collins, G., and Hilditch, T. P., *J. Chem. Soc.*, 246 (1933).
20. André, E., and François, T., *Compt. rend. Acad.*, **185,** 279 (1927).
21. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128,** 463 (1939).
22. Scott, N. D , Walker, J. F., and Hansley, V. L., *J. Am. Chem. Soc.*, **58,** 2442 (1936). Walker, J. F., and Scott, N. D., *J. Am. Chem. Soc.*, **60,** 951 (1938).
23. Morton, A. A., Laboratory technique in organic chemistry, New York, 120, fig. 63 (1938).
24. Baer, E., *Ind. and Eng. Chem., Anal. Ed.*, **16,** 399 (1944).
25. Toyama, Y., and Ishikawa, T., *J. Chem. Soc. Japan*, **59,** 1367 (1938).

CHEMICAL STUDIES ON THE TRANSFORMATION OF MOUSE EPIDERMIS BY METHYLCHOLANTHRENE TO SQUAMOUS CELL CARCINOMA*

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Previous studies (1) have shown that methylcholanthrene produced a notable decrease in the iron and calcium content of mouse epidermis, while the sodium, potassium, magnesium, and ascorbic acid contents were not appreciably affected. Moreover in a transplantable squamous cell carcinoma, derived from mouse epidermis by the application of methylcholanthrene, the calcium content was about 50 per cent lower and the potassium content 16 per cent less than that of hyperplastic (methylcholanthrene-treated) epidermis (2). For a more complete understanding of the events occurring in the process of epidermal carcinogenesis the magnesium and sodium contents of the transplantable carcinoma were determined.

EXPERIMENTAL

The methods for the determination of sodium (3), magnesium (1), and nucleoprotein phosphorus (NPP) (4), the basis of reference, in the transplantable carcinoma have been given. Since the carcinoma is vascular, the iron, due largely to hemoglobin, was removed by the procedure of Weeks and Todd (5) before magnesium was precipitated and determined polarographically as the hydroxyquinolate.

The characteristics and the method for sampling the transplantable tumor have been described (2). The carcinoma has now passed through thirty transplants and its morphology has remained practically constant. Portions of all tumors removed for analysis were fixed and stained for histological control, since necrosis has long been known to affect the mineral content of tissue (6). For the determination of sodium and magnesium pools of eight to twelve tumors were employed.

Sodium—Since whole blood is rich in sodium, it is necessary to correct for the amount of this metal present in tumors as blood sodium. The blood was removed, weighed, and ashed as before (2). Eleven samples of mouse whole blood contained an average of 0.047 mg. of iron per 100 mg. of blood, and five samples an average of 0.184 mg. of sodium per 100 mg. From the

iron content of a portion of the tumor mixture it is easy to calculate the amount of sodium due to blood alone if one assumes that most of the iron is derived from hemoglobin. Therefore on a pool of small tumors three determinations were carried out, nucleoprotein phosphorus, sodium, and iron. Since our previous investigations (2) had shown that small solid tumors (removed about 10 days after transplantation) were the most homogeneous chemically and morphologically, most of the work reported here was done on this type of tumor. A few large necrotic tumors were employed to ascertain the effect of degenerating tumor tissue on the sodium and magnesium contents.

The average sodium content of fifteen analyses of small solid tumors was 0.132 mg. per 100 mg. of tumor and the Na:NPP ratio was 0.95. All values

TABLE I

Effect of Necrosis upon Ratio of Metal to Nucleoprotein Phosphorus (NPP) of Carcinoma

Metal	Carcinoma	Average metal per 100 mg	Average blood metal per 100 mg	Average metal per 100 mg , corrected	Average NPP per 100 mg	Metal / NPP
		mg	*mg*	*mg*	*mg.*	
K	Solid	0.326	0.012	0.313	0.139	2.25
"	Necrotic	0.275	0.011	0 264	0.138	1.91
Ca	Solid	0.009			0.120	0.75
"	Necrotic	0.077				
Na	Solid	0.141	0.009	0.132	0.139	0.95
"	Necrotic	0.171	0.010	0.161	0.126	1.28
Mg	Solid	0.018			0.151	1.19
"	Necrotic	0.017			0.151	1.12

given here were corrected for blood sodium. This ratio of 0.95 was 22 per cent less than that of normal and hyperplastic epidermis. The necrotic tumors (freed of all gross necrotic tissue, but showing the latter histologically) had an average sodium content of 0.161 mg. per 100 mg. of tumor and an Na:NPP ratio of 1.28, which was about 9 per cent larger than that of normal epidermis and 26 per cent greater than that of the small solid tumors. The amount of blood sodium in the carcinomas, necrotic or solid, averaged about 6 per cent of the total.

Magnesium—The results for the magnesium analysis are expressed in the same manner as for sodium except that the Mg:NPP ratio was multiplied by 10. The magnesium content of small solid tumors (twenty-three analyses) varied from 0.017 to 0.021 mg. of Mg per 100 mg. of tumor (average 0.019) and had an Mg:NPP ratio of 1.19, which was 17 per cent less than that of normal epidermis. The necrotic tumors, freed of gross

necrotic tissue, contained 0.017 mg. of Mg per 100 mg. of tumor, and had a ratio of 1.12, which was 22 per cent less than that of normal epidermis and about 6 per cent less than that of the small solid type.

The care required in the sampling of tumor tissue, at least for inorganic analysis, is exemplified in Table I which shows the differences in the Ca, Mg, Na, and K ratios of nucleoprotein phosphorus of the small solid tumors and the large necrotic ones. It is apparent that the calcium and sodium ratios are increased in degenerating carcinomatous tissue, while potassium and magnesium are decreased.

<center>DISCUSSION</center>

The results of the investigations on epidermal carcinogenesis are shown graphically in Fig. 1. The time in days is plotted against the percentage of change of the metal-NPP ratios. As early as 10 days after one application of the carcinogen the iron and calcium ratios had dropped to about 50 per cent of the normal. There was a further reduction in the Fe:NPP ratio, while the diminution of the Ca:NPP ratio remained fairly constant. When the hyperplastic cells became carcinomatous, there was a further decrease in the calcium content. The Fe:NPP ratio shown on the graph for the carcinomas includes blood hemoglobin, and may be expected to show a greater drop when the latter is excluded. The possible significance of the calcium and iron diminutions has been discussed (2).

In a comparison of the chemical changes in the early stages of carcinogenesis with tissue found in a carcinomatous tumor it must be remembered that the cellular material analyzed in a carcinoma, which consists mainly of cancer cells together with some stroma, is more homogeneous than the cellular material presented by the several samples of epidermis obtained from the skin of eight to twelve different mice during the intermediate stages of carcinogenesis. In this latter material the skins of individual mice show considerable variation in the degree of epidermal hyperplasia. Moreover since cancer arises always in a sharply circumscribed area of a treated epidermis, there are considerable differences in one and the same skin of the degree of chemically precancerous hyperplasia. This means that the chemical analyses of such skin represent average values between the more advanced and the less advanced degree of epidermal hyperplasia. It is thus possible that the fall in the calcium and iron content is actually greater for the advanced stages than is indicated by the analyses, while the corresponding values for the less advanced stages are small.

On the other hand the magnesium, sodium, potassium, and ascorbic acid ratios appeared not to be significantly altered in hyperplastic epidermis. However, when the cells became malignant, there was the following decrease in the ratios, K:NPP 16 per cent, Mg:NPP 17 per cent, Na:NPP 22 per cent. The ascorbic acid-NPP ratio remained practically unchanged.

A comparison of the K:NPP/Na:NPP and the Ca:NPP/Mg:NPP ratios (Table II) further demonstrates that the alkalies are not appreciably altered, while the ratios of the alkaline earths are significantly changed in epidermal carcinogenesis. The K:NPP/Na:NPP ratio, except for a
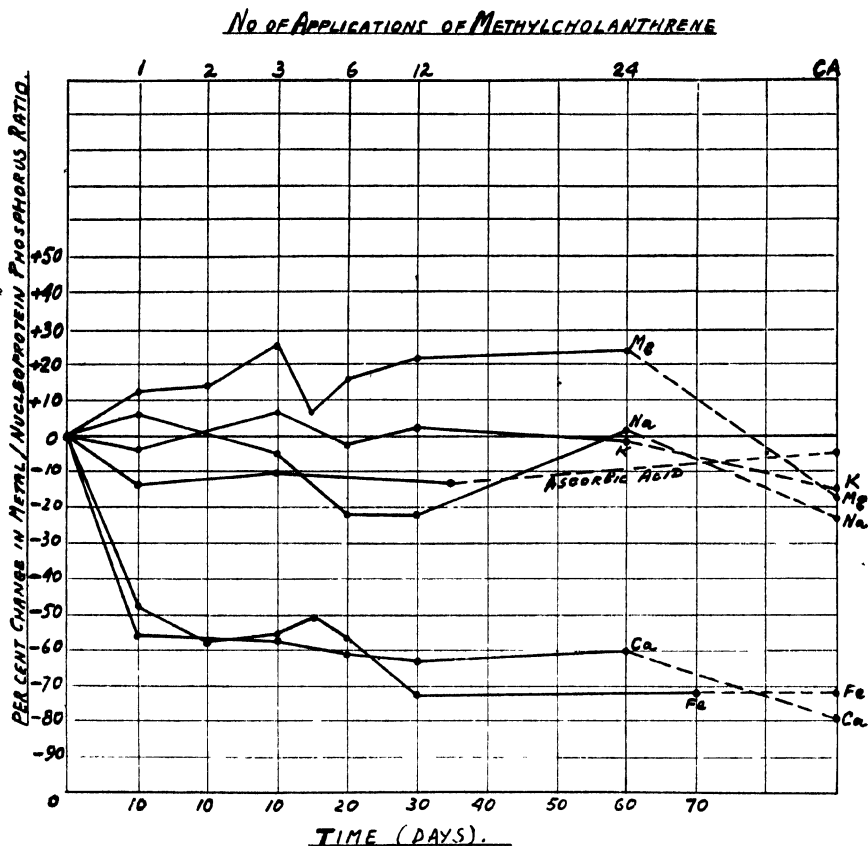
FIG. 1 Magnesium, sodium, potassium, calcium, iron, and ascorbic acid in epidermal carcinogenesis induced by methylcholanthrene CA represents carcinoma. Dotted lines (except for ascorbic acid from 35 to 60 days) are used to designate the change from late hyperplastic epidermis to carcinoma.

slight increase at 20 and 30 days, remained rather constant. On the other hand there was nearly a 58 per cent decrease in the Ca:NPP/Mg:NPP ratio after one application of the carcinogen, the ratio remaining fairly constant throughout the hyperplastic stage. There was a further drop of 23 per cent in the ratio when the cells became malignant.

In a review on the rôle of calcium on the boundary structure of cells, Reid (7) points out that this ion gives stability not only to cell surfaces, but also to intercellular substances. The observations of Cowdry and Paletta (8) on the decrease in intranuclear viscosity from normal to hyperplastic to carcinomatous cells may be partially explained by the decrease in the calcium content. Moreover the invasive properties of cancer cells may be explained on the same basis.

The chemical changes induced in mouse epidermis by a single application of methylcholanthrene, as shown in Fig. 1, reveal that the response to the carcinogen is rapid and chemically specific even at 10 days. Moreover Wicks and Suntzeff (9) have found that the ratio of total lipid to protein

. Table II

Ratio of Potassium to Sodium Nucleoprotein Phosphorus and of Calcium to Magnesium Nucleoprotein Phosphorus in Epidermal Methylcholanthrene Carcinogenesis

Treatment	No of paintings	Time after first treatment to killing of mice	$\dfrac{K:NPP}{Na.NPP}$	$\dfrac{Ca:NPP}{Mg:NPP}$
		days		
Normal . . .		10	2.29	2.57
Benzene-treated . _	3	10	2.18	2.19
Methylcholanthrene-treated	1	10	2.06	0.99
" .	3	10	2.55	0.85
"	6	20	2.90	0.85
"	12	30	3.03	0.75
"	24	60	2.24	0.82
Carcinoma.			2.36	0.63

nitrogen is also decreased by nearly 50 per cent 5 days after a single application of methylcholanthrene. The investigations of Cramer and Stowell (10) and of Simpson and Cramer (11) have demonstrated that one exposure of mouse skin to methylcholanthrene is able to produce cancer in about 40 per cent of the treated mice. Also Biesele and Cowdry (12) have found that diplochromosomes and even more greatly enlarged chromosomes are formed in epidermal cells on and after the 2nd day. Throughout the carcinogenic series, the frequency of enlarged chromosomes increased by about 8 per cent through the first 60 days, and in the carcinomas rose to over 50 per cent. All the above observations indicate that profound changes are induced very early by one application of methylcholanthrene sufficient in some animals to produce cancer without further treatment. The integration of the physical, histological, and chemical studies in our integrated series has been reviewed recently by Cowdry (13).

SUMMARY

The rôle of the minerals in epidermal carcinogenesis induced by methylcholanthrene has been investigated. A single application of the carcinogen produced a decrease in the calcium and iron content to about 50 per cent of the normal. Repeated treatments of the epidermis caused a further drop in the iron content, while the diminution of calcium induced by one application was not decreased appreciably. On the other hand the amount of potassium, sodium, magnesium, and ascorbic acid was not significantly altered by a single or by many applications of methylcholanthrene. The transplantable skin carcinoma, when small, solid, and practically free of necrotic tissue, contained less of all the minerals than did the normal and hyperplastic epidermis. However, necrotic tumor tissue had more calcium and sodium and less potassium and magnesium than did the small solid tumors. Care in sampling tumor tissue, at least for inorganic analyses, is stressed. Nucleoprotein phosphorus was used as a basis of reference for the amount of living tissue involved.

BIBLIOGRAPHY

1. Carruthers, C., and Suntzeff, V., *Cancer Res.*, **3**, 744 (1943).
2. Suntzeff, V., and Carruthers, C., *J. Biol. Chem.*, **153**, 521 (1944).
3. Suntzeff, V., and Carruthers, C., *Cancer Res.*, **3**, 431 (1943)
4. Carruthers, C., and Suntzeff, V., *J. Nat. Cancer Inst.*, **3**, 217 (1942)
5. Weeks, E. W., and Todd, J. R , *Ind. and Eng Chem., Anal. Ed.*, **15**, 297 (1943).
6. Shear, M. J , *Am. J. Cancer*, **18**, 924 (1933)
7. Reid, M. E., *Physiol. Rev.*, **23**, 76 (1943).
8. Cowdry, E. V., and Paletta, F. X., *Am. J. Path* , **27**, 335 (1941).
9. Wicks, L. F., and Suntzeff, V., *J. Nat. Cancer Inst.*, **3**, 221 (1942).
10. Cramer, W., and Stowell, R. E., *Cancer Res.*, **3**, 36 (1943).
11. Simpson, W. L., and Cramer, W., *Cancer Res.*, **3**, 604 (1943).
12. Biesele, J. J., and Cowdry, E V., *J. Nat. Cancer Inst.*, **4**, 373 (1944).
13. Cowdry, E. V., Biological symposia, Lancaster, **10**, 131 (1943).

INHIBITION OF BACTERIAL GROWTH BY d-LEUCINE*

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The present study was undertaken as one approach to an elucidation of the effective structure of the antibiotic, gramicidin (1). The results of investigations of this sort are expected to lead to improved antibiotics, and to experimental approaches to the paths of biological synthesis of protein.

A striking feature of gramicidin is its content of d-amino acids, notably d-leucine (2). The presence of d-amino acids in gramicidin seems sufficiently unusual to warrant studies of synthetic peptides containing the d-leucine residue. It appeared desirable to test d-leucine first, however, for antibiotic activity. The primary purpose of this paper is to report the unexpected finding of inhibition of bacterial growth by this unnatural isomer of a natural amino acid (Table I).

The organism chosen for testing was *Lactobacillus arabinosus* 17-5.[1] The culture named is being employed in this laboratory for assay of amino acids, as in the procedure of Shankman, Dunn, and Rubin (3), and was therefore convenient. The strain at hand had the added advantage of a known requirement for leucine, as well as ease of precise determination of growth by titration of the lactic acid produced. In most of the repetitions of the experiment on inhibition, of which Table I contains one example, differences could usually be seen between cultures containing the d-leucine and the control cultures after 1 day's incubation. The d-leucine tube was relatively unclouded with bacterial growth. Titration values at this stage were too close together for definite distinctions. The degree of growth at 3 days, in separate experiments, is presented in Table II.

It proved to be necessary to correct readings of acid production for the increase in the titratable acidity after autoclaving and incubating the medium. This increase can be accounted for by reaction of the amino acids and dextrose contained in the nutrient medium (4). The actual titration values for such tubes are presented in Table III.

Since brucine has a known toxicity (5) and since it was employed in the resolution of dl-leucine (6), it seemed necessary to check on the possibility of presence in the purified d-leucine of traces of brucine which might have

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Amino acid prefixes in this paper refer solely to configuration

[1] American Type Culture Collection, No. 8014.

carried through the process. Such traces might conceivably be physio-
logically significant although absent by chemical criteria. The utility of

TABLE I

Comparison of Leucine Isomers in Inhibition of Bacterial Growth

Addition to basal medium in tube	0 072 N acid produced		Average 0 072 N acid produced (corrected)
	cc.	cc	cc.
None .	3.76	3.88	3.53
50 mg. *l*-leucine .	3.84	3.74	3.24
50 " *d*-leucine	1.91	1.72	1.27
50 γ brucine sulfate	3.97	4.00	3.70
500 " " "	3.83	3.77	3.51
1 mg. gramicidin*	0.40	0.40	
10 " tyrothricin*	0.43	0.44	

* Kindly donated by Mr. Leo Wallerstein, the Wallerstein Laboratories, New
York.

TABLE II

Growth Inhibition in Series of Experiments

0 072 N acid produced in control (corrected)	0 072 N acid produced with *d*-leucine added (corrected)	$\dfrac{\text{Inhibited growth}}{\text{Control growth}}$
cc.	cc.	per cent
2.85	0.45	16
2.99	1.09	36
3.53	1.52	43
3.53	1.42	40
3.53	1.37	39
3.53	1.18	33
Average		35

TABLE III

Titratable Acidity after Autoclaving and Incubation

These tubes were not inoculated.

Addition to basal medium in tube	0 072 N acid produced	
	cc	cc.
None	0.21	0.29
50 mg. *d*-leucine	0.50	
50 " *l*-leucine	0.55	
50 " *dl*-leucine	0.48	0.54

50 mg. of *l*-leucine obtained by resolution with brucine was also tested and
this was found to be as devoid of inhibitory or stimulatory activity as
isolated leucine. It was also found that when 500 γ of brucine sulfate were

added to samples of complete basal medium growth was normal. Solutions containing this amount of brucine gave definitely positive nitric acid color tests. No effect can therefore be ascribed to hypothetical traces of brucine (Table I).

Materials and Methods

Formyl-dl-leucine—The formylation procedure of du Vigneaud, Dorfman, and Loring (7) for *dl*-cystine was found to be conveniently adaptable to *dl*-leucine in this study. 10 gm. of Merck's *dl*-leucine were dissolved in 150 cc. of 85 per cent formic acid. The temperature was brought to 55–60° and maintained there by regulated dropwise addition of 50 cc. of acetic anhydride. When this mass was cool, 25 cc. of water were added, and the clear solution was then concentrated under reduced pressure to about 40 cc. There were obtained 6.5 gm. of formylleucine, of which a sample melted at 112°. Another 2.6 gm. with a melting point of 115–117° were obtained from the mother liquor by evaporation and washing with chilled 1 N HCl, and then with chilled water.

d-Leucine and l-Leucine—The resolution procedure of Fischer and Warburg (6) was followed. Operations for the recovery of the leucine isomer from both brucinium formyl-*l*-leucinate and brucinium formyl-*d*-leucinate were as nearly identical as it was possible to make them. Brucine was absent in detectable amounts (nitric acid color test) from both formylleucines prior to further ether extraction of the aqueous solutions and subsequent conversion to purified leucines. A *d*-leucine sample in 20 per cent HCl had $[\alpha]_D = -15.1° \pm 0.5°$. Polarimetric values as an index of purity of leucine isomers should be interpreted cautiously. It has already been shown that values over the range 15.3° to 16.7° can be found for leucine samples which are pure by a sensitive physical criterion such as the heat of combustion (8).

Microbiological Procedures

The basal medium was essentially that of Kuiken et al. (9). Glutamic acid and tyrosine were, however, the natural isomers. No tomato eluate was included. The culture volume employed was 2.5 cc. Autoclaving was carried out for 20 minutes at 15 pounds steam pressure. Incubation occurred at 30° ($\pm 1°$) for 72 hours. A mixed methyl red-brom-thymol blue indicator was employed in titrations, which were conducted on the entire culture.

DISCUSSION

The above experiments demonstrate that *d*-leucine retards the growth of a strain of *Lactobacillus arabinosus* requiring *l*-leucine. Since the same relatively large quantity of *l*-leucine has practically no effect, inhibition is

isomeric in nature. The evidence therefore indicates that the d-leucine present in inhibitory experiments probably interferes with the uptake or utilization of l-leucine or l-amino acids by the organism.

This bears analogy to the mode of action of sulfonamides (10). The inhibition reported here, however, appears to depend upon a difference in spatial configuration rather than upon differences in chemical constitution.

The effectiveness of peptide antibiotics, such as gramicidin, tyrocidine, etc., may be attributable at least in part to the content of d-amino acids. The degree of this effect as well as the toxicity to mammals is undoubtedly influenced by the nature of other amino acids and linkages in the peptides.

The molecular ratio of d-leucine to l-leucine in the original medium in inhibition experiments is approximately 200:1. Gramicidin is more effective at a much lower ratio (Table I). The finding of measurable inhibition by d-leucine, however, makes it desirable to search for derivatives with special properties. Studies on applicability of related compounds and other organisms are under way

Grateful acknowledgment for *Lactobacillus arabinosus* cultures and advice on bacteriological techniques is made to Professor F. E. Nelson, of the Bacteriology Department. Thanks for technical assistance are also due Mr. Lee Lumpkin.

SUMMARY

Growth inhibition of a strain of bacteria by a d-amino acid isomer of a natural amino acid, l-leucine, required for growth of the same strain, has been demonstrated. The significance of this finding with regard to the peptide antibiotics has been discussed.

BIBLIOGRAPHY

1. Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **132**, 791 (1940).
2. Lipmann, F., Hotchkiss, R. D., and Dubos, R. J., *J. Biol Chem.*, **141**, 163 (1941).
3. Shankman, S., Dunn, M. S., and Rubin, L. B , *J. Biol. Chem.*, **150**, 477 (1943).
4. Frankel, M., and Katchalsky, A., *Biochem. J.*, **35**, 1034 (1941).
5. Henry, T. A., Plant alkaloids, Philadelphia, 530 (1939).
6. Fischer, E., and Warburg, O., *Ber. chem. Ges* , **38**, 3997 (1905).
7. du Vigneaud, V., Dorfman, R., and Loring, H. S , *J. Biol. Chem.*, **98**, 577 (1932).
8. Huffman, H. M., Fox, S. W., and Ellis, E. L., *J. Am. Chem. Soc.*, **59**, 2144 (1937).
9. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
10. Henry, R. J., *Bact. Rev.*, **7**, 175 (1943).

AN ELECTROPHORETIC STUDY OF SERUM AND PLASMA FROM NORMAL AND LEUCOSIS-AFFECTED CHICKENS*

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Poultry pathologists have observed that when chickens are affected with leucosis they may show one or more pathological manifestations. It is not yet known whether all the manifestations of the disease are caused by one or by different agents. Therefore, it was thought that a study of the blood serum and plasma proteins of normal and affected chickens might furnish information that would aid in clarifying the present state of knowledge pertaining to the different types of leucosis.

The study of the blood proteins was conducted in the Tiselius electrophoresis apparatus by the moving boundary method. Characteristic changes in the relative composition of protein components of serum have been noted in many pathological conditions in man and animals by this method (1–4).

Procedure

Chickens of the single combed white Leghorn breed from 15 to 18 weeks of age were used. They were usually fasted 24 hours previous to bleeding. Blood was collected from two to nine chickens and pooled. All the chickens were electrocuted and bled, and then examined for evidence of infection. For plasma samples, blood was collected in 0.01 volume of a 25 per cent solution of sodium citrate.

Total protein of each serum or plasma sample was determined by the refractive differential method of Siebenmann (5) and adjusted to a protein concentration of 2 per cent with buffer solution. The diluted sample was placed in cellophane tubing and dialyzed against three changes of buffer at 3°. The final dialysate was saved and used in filling the electrode vessels and cell. The buffer solution used on all samples consisted of diethylbarbituric acid and sodium hydroxide adjusted to pH 8.6 and ionic strength of 0.1.

Conductance measurements were made at 0° on the buffer and protein

after final dialysis. The Shedlovsky type of conductivity cell was used (2, 6) in a Dewar vessel containing crushed ice and water.

Electrophoresis was carried out in the modified Tiselius apparatus described by Longsworth and MacInnes (7). The schlieren scanning method of Longsworth (2, 8) was used in photographing the moving boundaries. Electrophoresis was carried out at 0° at a potential gradient of 6 to 7 volts per cm. The time varied from 10,000 to 12,000 seconds.

The first objective was to obtain the electrophoretic patterns for normal chicken serum and plasma and to establish the mobilities and ratio of normal protein components. After determination of the normals, a comparison was made with serum from chickens showing different pathological manifestations of the leucosis complex. Later, serum samples were obtained from chickens at intervals following the injection of a lymphoid tumor and also from chickens following the injection of this same tumor after inactivation. The inactivated preparation consistently failed to produce any manifestations of the disease.

Experiments and Results

Electrophoretic Patterns of Normal Chicken Serum and Plasma—Serum and plasma samples were obtained from apparently normal chickens which showed no symptoms or lesions of any disease. The electrophoretic diagrams shown in Figs. 1 and 2 are representative of the normal samples. Calculations were made from the patterns of the descending boundaries of four normal serum samples and two normal plasma samples. The average values obtained were as follows: mobility, albumin -5.9×10^{-5}, $\alpha_1 + \alpha_2$-globulin -4.7×10^{-5}, β-globulin -3.5×10^{-5}, fibrinogen -2.5×10^{-5}, γ-globulin -2.0×10^{-5}; percentage composition, albumin 46.8, $\alpha_1 + \alpha_2$-globulin 17.9, β-globulin 11.3, fibrinogen 13.5, γ-globulin 19.4; composition ratios, (albumin)/(globulin) 1.00, $(\alpha_1 + \alpha_2)$/albumin 0.39, β/albumin 0.25, ϕ/albumin 0.30, γ/albumin 0.43. The total protein in normal serum varied from 2.19 to 3.74 gm. per 100 ml.

A very pronounced γ-globulin disturbance is present in the patterns of all chicken serum and plasma. It appears as a tall spike on all descending patterns and as a small peak on ascending patterns. A similar disturbance in the β-globulin in human serum has been observed by Longsworth et al. (2, 9). It is attributed to convection resulting from reaction in the neighborhood of the boundary following electrophoretic separation of the constituents. Longsworth has shown that at least one of the β-globulins of normal serum has an affinity for lipid material which migrates with the β-globulin. Abramson et al. (10) have discussed the physics of the β anomaly and other possible explanations.

Electrophoretic Patterns of Serum from Leucosis-Affected Chickens[1]—Serum samples for electrophoretic study were obtained from typical cases of neurolymphomatosis, visceral lymphomatosis, osteopetrotic lymphomatosis, and erythrogranuloblastosis. Electrophoretic patterns of these samples (Figs. 3 to 6) show the usual peaks for the four normal proteins and an additional peak which is designated as the L component. The new component is closely associated with γ-globulin and moved with that component in electrophoresis of the neurolymphomatosis serum. Since the L component is apparently masked on the descending patterns by the
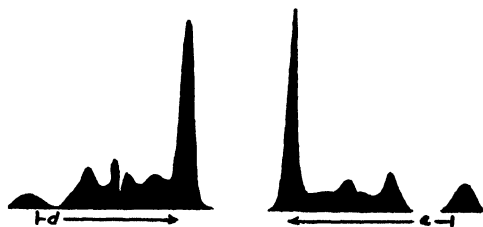
FIG. 1. Sample 429. Normal chicken serum. Electrophoresis for 12,000 seconds at 6.42 volts per cm.
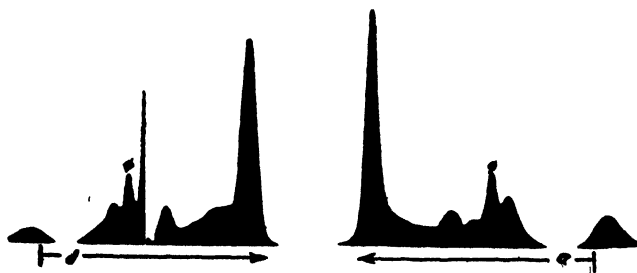
FIG. 2. Sample 450. Normal chicken plasma. Electrophoresis for 12,000 seconds at 6.8 volts per cm.

boundary disturbances, calculations of mobility and relative composition were made from the ascending patterns. According to Longsworth and MacInnes (13), close agreement of mobility on ascending and descending patterns may be obtained by using an equation which includes the ratio of area of the ascending peak to the area of the descending peak and correction for the ε-boundary. This equation could be applied only to those patterns in which the area of the L component could be measured on both

[1] The terms used in describing the types of the disease were adopted by a conference of investigators held at the United States Regional Poultry Research Laboratory (11, 12).
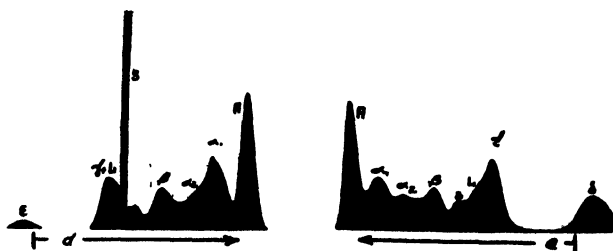
FIG. 3. Sample 230. Composite serum from chickens showing visceral lymphomatosis. Electrophoresis for 12,000 seconds at 6.95 volts per cm.
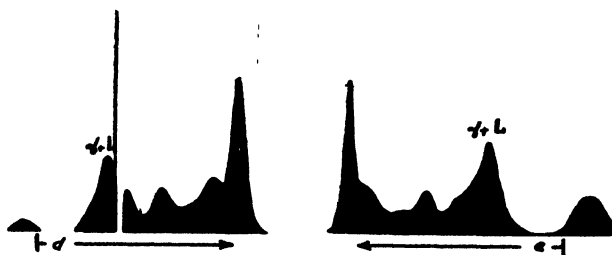
FIG. 4. Sample 233. Composite serum from chickens showing neurolymphomatosis. Electrophoresis for 12,000 seconds at 6.64 volts per cm.
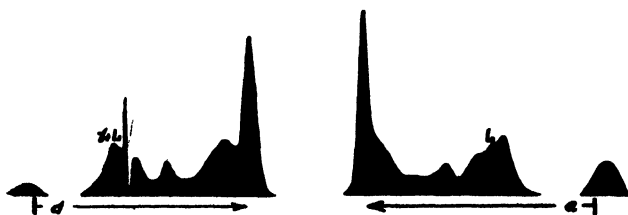
FIG. 5. Sample 234. Composite serum from chickens showing osteopetrotic lymphomatosis. Electrophoresis for 12,000 seconds at 6.69 volts per cm.
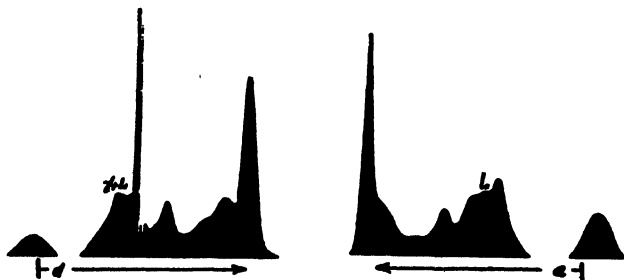
FIG. 6. Sample 236. Composite serum from chickens showing erythrogranuloblastosis. Electrophoresis for 12,100 seconds at 6.53 volts per cm.

ascending and descending patterns. The average mobility of the L component calculated from ascending patterns was -2.9×10^{-5}. The "corrected" value from two ascending patterns, -2.65×10^{-5}, closely agreed with the more accurate mobility, -2.55×10^{-5}, obtained from a later experiment in which the L component was visible on the descending patterns.

Included in Table I are relative compositions of components of all normal and pathological serums studied. A comparison of normal serum with visceral, neuro-, and osteopetrotic lymphomatosis, and erythrogranuloblastosis shows significant changes in composition. There was an average decrease in albumin content from a normal 46.0 to 32.2 per cent; the α-globulin increased from 13.1 to 20.0 per cent; the β- and γ-globulins showed no significant differences. Changes in concentrations of the components resulted in the alteration of the albumin-globulin ratio from 0.87 to 0.46 and the α-globulin-albumin ratio from 0.27 to 1.01.

In one sample, neurolymphomatosis, the L component moved with the γ-globulin, which accounted for 37.2 per cent of the total protein. The L component represented an average of 10.5 per cent of the total protein.

Electrophoretic Patterns of Serums Obtained at Intervals after Injection with Active Transmissible Tumor—Following the observation of the presence of the L component in serum from chickens showing all the different manifestations of the avian leucosis complex, the next logical step was to correlate the appearance and development of symptoms of leucosis with the appearance of the L component in the blood. This study was performed on the blood of chickens affected with a transmissible lymphoid tumor. The strain was the one recovered by Olsen (14) and termed "R. P. L. 12" by the Regional Poultry Research Laboratory. Tumors are produced in the muscle tissues of chickens within 5 days after injection and in the visceral organs after a longer period.

Pectoral tumors obtained from three chickens were minced and an equal volume of 0.85 per cent sodium chloride added. 1 ml. of this suspension was injected intramuscularly into susceptible chickens. Composite serum samples for electrophoresis were obtained from three chickens at 1 day intervals after injection. The last was collected on the 19th day.

The chickens killed on the 3rd day after injection showed no symptoms or internal lesions. However, the electrophoretic patterns of the serum (Fig. 7) show the L component present. The data shown in Table I indicate a change in the relative composition of protein components.

Small tumors were observed in the pectoral muscle in two of the three chickens examined on the 5th day. An increase in γ-globulin to 31.8 per cent of the total protein indicates that the L component moved as a fraction of γ-globulin. The albumin content decreased from a normal 46.0 to 38.5 per cent.

From the 7th to the 19th day, tumors in the pectoral muscles increased

TABLE I

Relative Composition* of Protein Components in Serum and Plasma from Normal and Leucosis-Affected Chickens

Sample No.	Origin of sample	Total protein (gm. per 100 ml.)	Albumin (gm. per 100 ml.)	Concentration, per cent total area							Composition						
				Albumin	α_1	α_2	β	ϕ	L	γ	A/G	α_1/A	α_2/A	β/A	ϕ/A	L/A	γ/A
429	Normal	2.68	1 27	47.4	9.0		14.4			19.2	1.34		0.16	0.25			0.33
348	"	2.19	0 91	41.7	8.3	3.8	12.8			32.5	0.72	0.20	0.11	0.31			0.78
362	"	3 13	1 45	47.8	8.5	7 8	14 8			21.1	0.92	0.18	0.16	0 31			0.44
450	" †	2 48	1.17	47 2	15 0		10.9	14 1		13 7	1 22	0.32		0.22	0.30		0.28
421	Transmissible tumor†	3 25	0 91	28.0	9.0	6.2	8 6	27.4		20.9	0.39	0.31	0.22	0.31	0.98		0 75
230	Visceral lymphomatosis	3 15	0.90	28 5	17.4	11.3	14.3		11.8	16.7	0 40	0 62	0.40	0.50		0.42	0.59
233	Neurolymphomatosis	3.28	0.99	30 1	14.6	4 8	13 2			37.2	0.43	0 48	0.16	0.44			1.23
234	Osteopetrotic lymphomatosis	3 04	1 05	34 7	20.4	6.9	13 7		9.2	17.1	0 53	0.59	0.22	0.34		0.27	0.49
236	Erythrogranuloblastosis	3.18	1 06	33.2	15.2	3.6	14.1		10.5	23.4	0.50	0.46	0.10	0.43		0.32	0.76
320	3 days after injection with tumor	2.91	1 25	43.1	16.5		10.3		13.1	17.1	0.76	0.39		0 24		0.30	0.40
329	5 " " " " "	3.58	1 38	38.5	14 0		15.7			31.8	0.63	0.36		0.41			0.83
344	7 " " " " "	3 17	1 04	32.9	10.1	9.9	15.9		10 7	20.5	0 49	0.31	0.30	0.48		0.33	0.62
336	9 " " " " "	2 93	1 02	35.0	12 4		14.9			26 7	0.54	0.43		0.51			0.92
349	13 " " " " "	2 30	0.84	36.3	12 6	10 0	12.1		10.7	18.4	0 57	0.35	0.27	0.33		0.29	51
356	17 " " " " "	3.18	1.26	39 7	12 1		14 6		10.5	23.2	0 66	0.31		0.37		0.26	58
357	19 " " " " "	3.12	1 19	38 3	10.3	6.7	11 5			33.2	0.62	0 27	0 18	0.30			0.87
330	7 " " " " inactivated tumor	3.13	1 31	41.9	18.2		14.2			25.8	0 72	0.43		0.34			0.62
353	15 days after injection with inactivated tumor	3.44	1.41	41.0	7.3	5.4	15 8		11.9	18.6	0.70	0.18	0.16	0.36		0.29	0.45
361	19 days after injection with inactivated tumor	2.86	1 25	43.6	6.5	6.7	13 0			30.3	0.77	0.15	0.15	0.30			0.69

A = albumin; G = globulin.
* Calculations from ascending patterns, uncorrected.
† Plasma.

in size in all chickens and intradermal tumors also were present. The L component was present in all ascending patterns, as a separate fraction in the sample of the 7th, 13th (Fig. 8), and 17th days, and as a component in γ-globulin in samples of the 9th and 19th days (Fig. 9). The albumin content dropped to a minimum of 32.9 per cent on the 7th day. The albumin-globulin ratio showed a gradual decrease from 0.76 on the 3rd day to a minimum of 0.49 on the 7th.
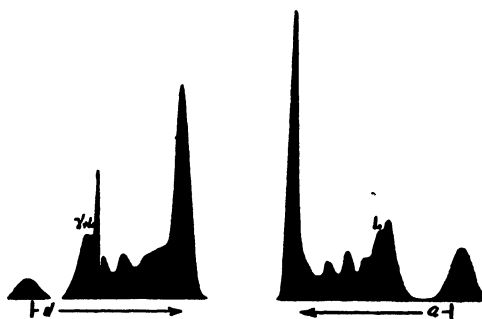
Fig. 7. Sample 320. Composite serum from chickens 3 days after injection with transmissible tumor. Electrophoresis for 10,220 seconds at 6.19 volts per cm.
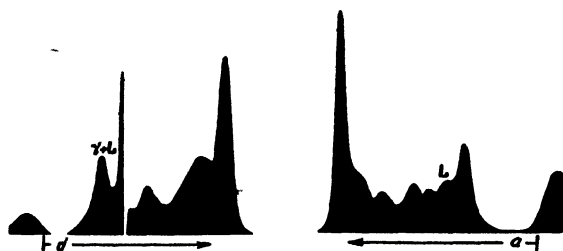
Fig. 8. Sample 349 Composite serum from chickens 13 days after injection with transmissible tumor Electrophoresis for 12,260 seconds at 6.33 volts per cm.

Calculations on the ascending patterns show the average mobility of the L component to be -3.0×10^{-5} as compared with the average -2.9×10^{-5} in the earlier studies of serums from chickens showing different types of the avian leucosis complex.

The early appearance of the L component in the serum and the early shift in composition indicate that there is a rapid change in the blood serum proteins of chickens affected with the transmissible tumor agent.

Electrophoretic patterns of plasma from apparently normal chickens (Fig. 2) were compared with the patterns of plasma from chickens 19 days after injection with a tumor-producing agent (Fig. 10). The comparison

revealed the same decrease in the albumin-globulin ratio that was observed in serum (Table I). The normal plasma contained 14.1 per cent fibrinogen; the increase to 27.4 per cent of this component in the leucosis plasma indicates that the L component moved at approximately the same rate as fibrinogen.

Electrophoretic Patterns of Serums Obtained at Invervals after Injection with Inactivated Transmissible Tumor—The inactivated material was prepared from the tumor-producing agent described in the section above by
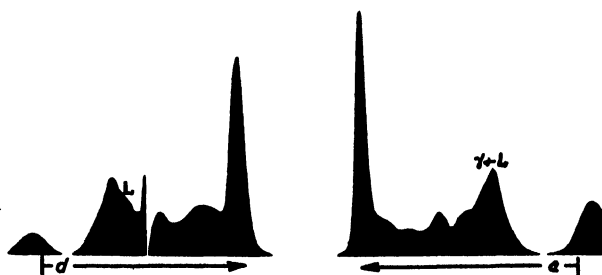
FIG 9. Sample 357. Composite serum from chickens 19 days after injection with transmissible tumor. Electrophoresis for 12,000 seconds at 6.39 volts per cm.
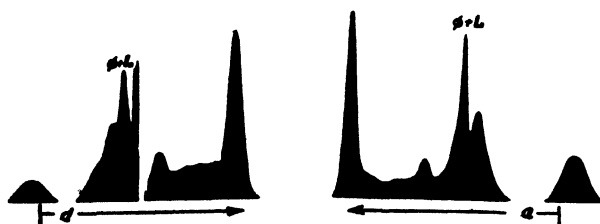
FIG. 10. Sample 421. Composite plasma from chickens injected with transmissible tumor. Electrophoresis for 12,000 seconds at 6.61 volts per cm.

rapidly freezing to −76° and thawing twice. As in the previous experiment, a composite serum sample for electrophoretic study was obtained from three chickens at 1 day intervals after injection. Tissues taken at necropsy showed no evidence of tumor formation in any of the chickens injected with the inactivated material.

The electrophoretic pattern of the serum sample collected on the 7th day after injection (Fig. 11) is apparently normal. The 15th day sample (Fig. 12) shows the presence of the L component, accounting for 11.9 per cent of the total protein in the sample. There is close agreement between the mobility value −2.6 × 10^{-5} of the L component in this serum with that found in serum samples from affected chickens, which was −2.5 × 10^{-5}.

The electrophoretic patterns of the sample collected on the 19th day after injection (Fig. 13) show the L component present and moving with the
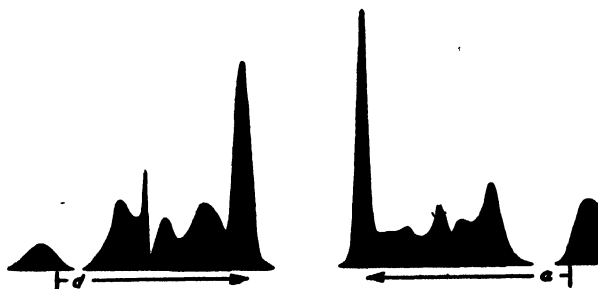
FIG. 11. Sample 330. Composite serum from chickens 7 days after injection with inactivated transmissible tumor. Electrophoresis for 12,620 seconds at 5.88 volts per cm.
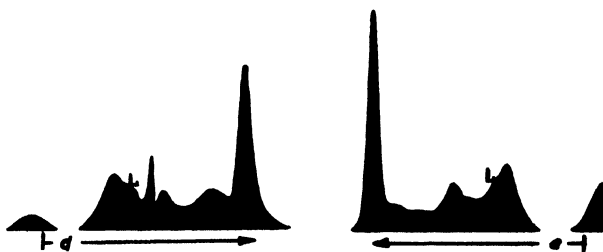
FIG. 12. Sample 353. Composite serum from chickens 15 days after injection with inactivated transmissible tumor. Electrophoresis for 12,330 seconds at 6.47 volts per cm.
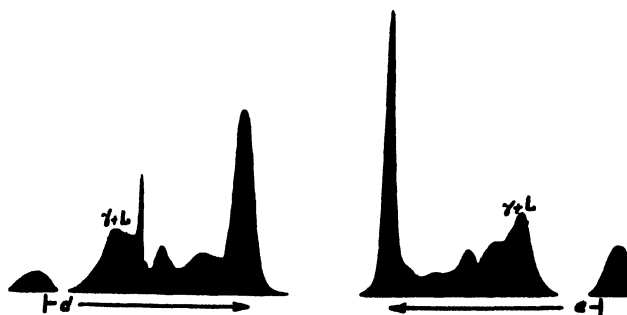
FIG. 13. Sample 361. Composite serum from chickens 19 days after injection with inactivated transmissible tumor. Electrophoresis for 12,000 seconds at 6.64 volts per cm.

γ-globulin, as evidenced by an increase of that component to 30.3 per cent of the total protein.

TABLE II

Mobility and Relative Composition* of Protein in Three Types of Chicken Serum

Sample No	Type of serum	Mobilities, u × 10^5					Total protein, gm. per 100 ml.	Albumin, gm. per 100 ml.	Concentration, per cent total area					Composition				
		Albumin	α1+α2	β	L	γ			A	α1+α2	β	L	γ	A/G	$\frac{\alpha_1+\alpha_2}{A}$	$\frac{\beta}{A}$	$\frac{L}{A}$	$\frac{\gamma}{A}$
429	Normal	5 7	4 5	3 5		1.9	2 68	1 55	57.9	15.5	9.3		17.2	1.37	0.27	0 16		0.30
357	Transmissible tumor	6 0	4 8	3 6	2 6	2 1	3.12	1 24	39.9	21 9	10 1	9 2	18.9	0 66	0 55	0 25	0 23	0 47
353	Inactivated transmissible tumor	6 0	5 0	3 5	2 6	2 0	3 44	1.46	42.3	17 8	11 3	9 5	18.9	0 73	0.42	0 27	0 23	0 45

A = albumin; G = globulin.

* Calculations from descending patterns.

ratio values of the proteins in a composite serum sample from normal chickens, one from chickens injected with active tumor agent, and one from chickens injected with inactivated transmissible tumor agent. These values, calculated from the descending patterns, are more accurate than those calculated from the ascending patterns and shown in Table I.

<div align="center">DISCUSSION</div>

Electrophoretic analysis of the proteins in serums from chickens affected with leucosis reveals the presence of a protein component which is not found in normal chicken serum. The new component is closely associated with γ-globulin and has been designated by the letter L.

The L component in blood serum samples taken from chickens that showed gross lesions of leucosis represents approximately 10 per cent of the total serum proteins. It does not seem likely that the protein of the leucosis agent would be present in blood serum in such a high concentration. By crude electrophoretic methods, Lee and Wilcke (15) studied the filtrate of a tumor-producing material and reported that the causative agent of fowl leucosis migrated to the cathode at pH 4.01 to 6.01 and to the anode at pH 7.01 to 9.01. They found that the causative agent was inactivated at pH 4 and 9.

Previous electrophoretic studies (16–19) of blood serums have shown that the antibody proteins are closely associated with γ-globulin and migrate as a component of that globulin or as a separate fraction of a mobility slightly greater than that of γ-globulin. Tiselius and Kabat (3) found that the pneumococcus carbohydrate-precipitating serum antibody, produced in the horse, migrated as a new component. A similar antibody, produced in the rabbit, appeared as an addition to the normal γ-globulin. The new component was no longer present in horse serum after the antibody had been removed by homologous type-specific polysaccharide or by a heavy suspension of specific pneumococci. In the rabbit antiserum, the amount of γ-globulin increased to 56 per cent of the total protein as compared with 17 per cent in normal serum. Pneumococcus antibody was shown by van der Scheer and his associates (4) to migrate with the γ-globulin component of serum from fourteen out of fifteen hyperimmunized horses. In the one exception, shortly after hyperimmunization, the antibody protein migrated as a separate component between β- and γ-globulins. The mobility was -2.1×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} in a 0.02 M phosphate buffer solution of pH 7.6. This mobility is very close to that calculated for the L component in serum from leucosis-affected chickens. The T component demonstrated in diphtheria and tetanus antitoxins from the horse appeared between β- and γ-globulins (20). Shapiro and his associates (21) separated a viscous protein from the blood plasma of a patient

with multiple myeloma; the pattern of the extra protein appeared between β- and γ-globulins and moved with fibrinogen.

It is postulated that the new L component described herein is of the nature of an antibody protein and occurs in all types of the avian leucosis complex. Also, it may be produced by the injection of an inactivated transmissible tumor.

The occurrence of the L component in serum after the injection of inactivated tumor material suggests further investigations into its immunological and diagnostic possibilities.

Since the electrophoretic patterns of chicken serum show that the L component is closely associated with γ-globulin, it was thought possible that the salting-out of the γ-globulin might also remove the antibody protein. This was attempted by adding 30 per cent sodium sulfate to an equal volume of serum from chickens showing leucosis tumors. The precipitated globulin was centrifuged, washed, dissolved, and dialyzed against 0.85 per cent sodium chloride solution. The protein solution was diluted to a protein concentration of 2 per cent. The electrophoretic pattern of this fraction had the appearance of pure γ-globulin. There was no separation of the L component.

SUMMARY

A comparative electrophoretic analysis by the moving boundary method has been made of the proteins in serum and plasma from normal chickens and from chickens affected with avian leucosis.

A new component, designated the L component, was found in serums from chickens affected with the several forms of leucosis. The new protein component had a mobility -2.55×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and represented approximately 10 per cent of the total protein in the serum. There were no significant differences found in the electrophoretic patterns of serums that would distinguish any one type of leucosis from another.

The relative composition of the components in leucosis serum (Table I) as compared with the normal showed a decrease in albumin and an increase in α-globulin. The electrophoretic patterns in which the new component failed to migrate as a separate peak showed a very high concentration of γ-globulin. This fact would indicate a close relationship between the L component and γ-globulin.

A series of blood samples drawn from chickens at intervals after injection with an active transmissible tumor agent showed the presence of the L component as early as the 3rd day. Chickens injected with the inactivated tumor agent also showed the presence of the L component but not until the 15th day after injection.

Attempts to separate the new protein component from γ-globulin by a salting-out method were unsuccessful.

The presence of the L component in the serum from leucosis-affected chickens may have immunological and diagnostic significance.

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BIBLIOGRAPHY

1. Gray, S. J., and Barron, E. S. G., *J. Clin. Invest.*, **22**, 191 (1943).
2. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med*, **70**, 399 (1939).
3. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, **69**, 119 (1939).
4. van der Scheer, J., Lagsdin, J. B., and Wyckoff, R. W. G., *J. Immunol.*, **41**, 209 (1941).
5. Siebenmann, C., *Biochem. J.*, **31**, 205 (1937).
6. Shedlovsky, T., *J. Am. Chem. Soc.*, **54**, 1411 (1932).
7. Longsworth, L. G., and MacInnes, D. A., *Chem. Rev.*, **24**, 271 (1939).
8. Longsworth, L. G., *Ann. New York Acad. Sc.*, **39**, 187 (1939).
9 Longsworth, L G., and MacInnes, D. A., *J. Exp. Med.*, **71**, 77 (1940).
10. Abramson, H. A., Moyer, L. S., and Gorin, M. H., Electrophoresis of proteins, New York, 185-186 (1942).
11. Cottral, G. E., Collaborators conference, 1941, Regional Poultry Research Laboratory, East Lansing, Michigan.
12. Jungherr, E., *Am. J. Vet. Res.*, **2**, 116 (1941).
13. Longsworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
14. Olsen, C., Jr., *Cancer Res.*, **1**, 384 (1941).
15. Lee, C. D., and Wilcke, H. L., *J. Am. Vet. Med. Assn.*, **94**, 178 (1939).
16 San Clemente, C. L., *Am. J. Vet. Res.*, **3**, 219 (1942).
17. San Clemente, C. L., and Huddleson, I. F., *Michigan Agr. Exp. Sta.*, *Bull. 182*, 3 (1943).
18. Tiselius, A., *Biochem. J.*, **31**, 1464 (1937).
19. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H., *J. Immunol.*, **39**, 65 (1940).
20. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H., *J. Immunol.*, **41**, 349 (1941).
21. Shapiro, S., Ross, V., and Moore, D. H., *J. Clin. Invest.*, **22**, 137 (1943).

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THE METABOLISM IN VITRO OF TYROSINE BY LIVER AND KIDNEY TISSUES OF NORMAL AND VITAMIN C-DEFICIENT GUINEA PIGS*

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The recently discovered rôle of ascorbic acid in controlling the metabolism of phenylalanine and tyrosine furnishes a new approach to the as yet unsettled question of the biochemistry of the vitamin in the animal organism. It seems reasonable to believe that the elucidation of the ascorbic acid-amino acid relationship may throw light on other aspects of the vitamin function. The converse is, of course, also true.

The amino acid problem may be summarized briefly (1). The ingestion of extra amounts of phenylalanine or tyrosine by the vitamin C-deficient guinea pig (2, 3) or premature infant (4) leads to the urinary excretion of a high percentage of partial metabolites of the latter amino acid. With the administration of relatively small amounts of the vitamin the metabolites are no longer present in the urine. That this is a specific property of the antiscorbutic substance became evident when the isomeric compound, d-isoascorbic acid, in equimolar quantities failed to abolish metabolite excretion (2).

The search for the metabolic system responsible for the ascorbic acid effect would be greatly facilitated if it could be shown that the vitamin action resides in or is more prominent in a single tissue. That this objective has been achieved, at least partially, is evident in the results of the present investigation, which demonstrate the *in vitro* oxidation of l-tyrosine by liver slices from normal guinea pigs and the inability of the same tissue from vitamin C-deficient animals to exhibit similar metabolic activity. Kidney tissue when studied in the same fashion showed only a little tendency to oxidize the amino acid, although also reflecting the state of vitamin C nutrition of the test animal.

EXPERIMENTAL

For these experiments, 300 to 400 gm. guinea pigs were maintained on a vitamin C-free basal diet of ground and aerated Purina rabbit chow (com-

plete ration). Some of the animals received in addition mixed fresh green foods or crystalline ascorbic acid. The scorbutic animals, all of which exhibited the characteristic symptoms, were used after 15 to 20 days on the basal diet. Individual animals, without having been fasted, were stunned by a blow at the back of the head and killed by bleeding from the severed jugular veins.

The *in vitro* respiration of slices from both the liver and kidneys was determined in the case of each animal, the Warburg-Barcroft technique being used. The modification of the Warburg vessel designed in this laboratory and described by Marsh (5) was used in order that the oxygen and carbon dioxide might be determined on the same pieces of tissue. The tissue slices were suspended in 1 ml. of Ringer-phosphate buffer, pH 7.4, in one compartment of the vessel. In the corresponding side arm was placed 1 ml. of the Ringer-phosphate solution containing 1 mg. (5.52 micromoles) of *l*-tyrosine. For the purpose of determining the basal consumption of oxygen and production of carbon dioxide, tyrosine was omitted from the solution in the side arm of an equal number of vessels. In the remaining compartment and side arm alkali and acid respectively were placed. After an equilibration period of 12 minutes the additional Ringer-phosphate solutions were emptied into the tissue compartment. Oxygen consumption measurements were made at intervals for a period of 3 hours, after which the total carbon dioxide of each flask was determined by thorough mixing of the acid into both vessel compartments. Removal of the tissues and subsequent drying then permitted the calculation of the Q_{O_2} and Q_{CO_2} (c.mm. per hour per mg. of dry tissue). Two, three, or four flasks were used for each experimental condition.

In view of the conflicting results obtained when the oxygen consumption of normal and scorbutic tissues has been compared (see (6)) the results derived from all control flasks of these experiments have been assembled in Table I. The Q_{O_2} value of the scorbutic liver slices is 28.2 per cent higher than that for the normal liver slices. These results are therefore in agreement with the findings of Stotz *et al.* (6), the increased oxygen consumption of the scorbutic tissue being obtained in this case without the administration of glucose at the beginning of the fasting period or the inclusion of glucose in the medium, the modifications employed by the latter investigators. The absence of glucose perhaps accounts for the failure to observe a similar increase in carbon dioxide production, the difference between the means for that gas recorded in Table I having been found not significant by the methods of statistical analysis. This finding is in contrast with that previously recorded (6). In the same communication results have been described showing that the onset of scurvy does not alter the oxygen consumption or carbon dioxide production of kidney cortex slices. Our

own results are in agreement, for the differences recorded in Table I are not statistically significant.

With the behavior of the control tissues in mind the results obtained when tyrosine is present as substrate may be considered. In order to present the results as completely as possible it has been of value to make comparisons in two ways, particularly since the Q_{O_2} value alone is not entirely informative. We have therefore deducted the calculated basal oxygen of the tyrosine-containing experimental flasks, using the average Q_{O_2} obtained with the control flasks. The remainder of the oxygen uptake then has been calculated in terms of the ratio of atoms of oxygen to moles of tyrosine present. A similar calculation has been made for the carbon dioxide production. That the exactness of such calculations is necessarily limited

TABLE I

Respiration of Normal and Scorbutic Guinea Pig Liver and Kidney Slices

			No of guinea pigs	Q values		
				Range	Mean	Difference
						per cent
Liver	Q_{O_2}	Normal	22	1.77– 2.67	2.16 ± 0.198	
		Scorbutic	8	2.15– 3.86	2.77 ± 0.569	+28.2
	Q_{CO_2}	Normal	22	1.61– 3.11	2.18 ± 0.425	
		Scorbutic	8	1.94– 2.95	2.43 ± 0.376	+11.5
Kidney	Q_{O_2}	Normal	22	5.90– 9.39	8.04 ± 1.07	
		Scorbutic	8	7.05–11.56	9.24 ± 1.72	+14.9
	Q_{CO_2}	Normal	22	5.40– 9.89	6.78 ± 1.00	
		Scorbutic	8	5.82–10.44	8.09 ± 2.20	+19.3

The tissues were shaken 3 hours in Ringer-phosphate, pH 7.4, in Marsh vessels in an atmosphere of air. Q = c.mm. per hour per mg. of dry tissue. Standard deviations are included with the mean values.

is well known. For example, the recent discussion by Laser (7) emphasizes the difficulties involved. For this reason the Q_{O_2} and Q_{CO_2} values as well as the ratios have been included in the summaries.

The oxygen consumption of liver slices from a normal and a scorbutic guinea pig is illustrated in Fig. 1, in which the c.mm. of oxygen per mg. of tissue (dry weight) are plotted against time. It is evident that the addition of *l*-tyrosine to the flasks containing the normal liver tissue produces a marked increase in the oxygen uptake. In the case illustrated the extra oxygen represents an average increase of 38.3 per cent above the control level. A similar situation does not occur with the scorbutic liver, for as shown in the right-hand graph the curve obtained when tyrosine is present may almost be superimposed upon the curve for the control values.

This difference between the ability of normal and scorbutic slices to oxidize tyrosine is further demonstrated in the summary values of Table II. It is apparent in the case of normal liver that for each mole of tyrosine present in the reaction vessel 1 atom of extra oxygen is consumed. On the other hand, with liver slices from vitamin C-deficient guinea pigs the ratio of atoms of oxygen to moles of tyrosine is less than 0.2. In fact, from the four different scorbutic animals used six of the ten values obtained were less than 0.1 atom per mole of tyrosine.

That the ability of the liver cells to oxidize tyrosine is not permanently lost with the occurrence of scurvy may be demonstrated. To deficient
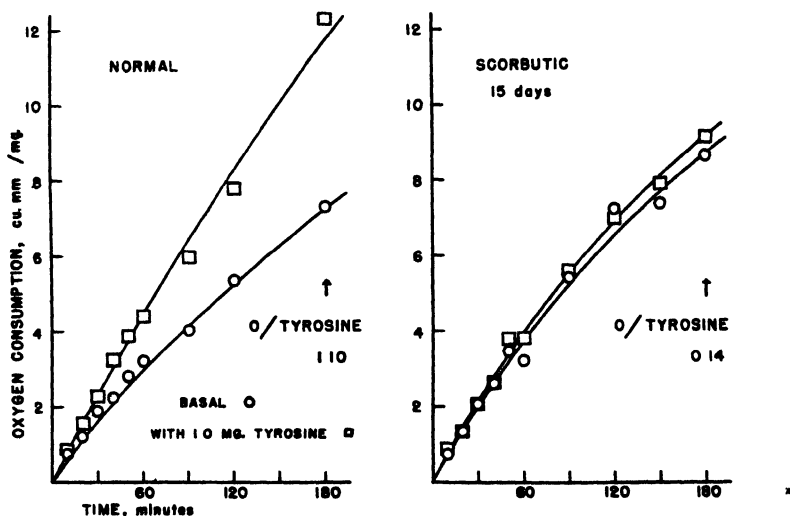
FIG. 1. The oxygen consumption of liver slices from a normal and a scorbutic guinea pig. Each point represents the average of duplicate or triplicate determinations.

guinea pigs comparable to those of Column 3 were administered subcutaneously 20 mg. of ascorbic acid each of 6 days immediately prior to sacrifice. The values of Column 4 are in direct contrast with those obtained with the scorbutic animals and are entirely like those obtained with the normal animals. That administration of ascorbic acid was efficacious is also observed in the Q_{O_2} of the control tissues, which is almost identical with that derived from normal rather than from scorbutic animals. On the other hand, similar injection of the vitamin into normal animals did not alter the oxygen consumption (Column 5). Thus added evidence of the dependence of tyrosine oxidation upon a normal level of ascorbic acid in the liver is obtained.

In Table III, which summarizes the values for carbon dioxide production for the same animals represented in Table II, it is apparent that this metab-olite reflects the picture obtained with oxygen as far as vitamin deficiency is concerned. However, the actual yield of carbon dioxide is considerably

TABLE II

Tyrosine Oxidation by Liver Slices

The results obtained with four guinea pigs are represented in each group.

(1)	Normal (2)	Scorbutic (3)	Ascorbic acid injected	
			Scorbutic (4)	Normal (5)
Control flasks, without substrate				
Tissue dry weight, average, *mg.*	19.5	19.8	13.8	17.2
Q_{O_2}, range	2.04–2.41	2.03–3.78	2.03–2.30	2.11–2.33
" average .	2.24	2.83	2.14	2.20
With 5.52 micromoles tyrosine				
Tissue dry weight, average, *mg.*	14.1	19.8	13.4	15.5
Q_{O_2}, range	3.47–4.25	2.37–3.83	3.70–5.33	3.83–4.65
" average	3.98	2.91	4.42	4.12
$\frac{O}{Tyrosine}$, range	1.00–1.40	0 –0.70	0.99–1.84	1.20–1.61
" average	1.14	0.188	1.44	1.41

TABLE III

Carbon Dioxide Production by Liver Slices

The results obtained with four guinea pigs are represented in each group.

		Normal	Scorbutic	Ascorbic acid injected	
				Scorbutic	Normal
Control flasks, without substrate	Q_{CO_2}, range	1.86–3.12	1.83–2.89	2.01 –2.32	1.47 –1.89
	" average	2.42	2.31	2.18	1.75
With 5.52 micro-moles tyrosine	" range	2.47–4.92	2.08–2.97	2.31 –2.92	2.22 –2.89
	" average	3.18	2.41	2.58	2.51
	$\frac{CO_2}{Tyrosine}$, range	0 –0.545	0 –0.292	0.062–0.179	0.150–0.536
	" average	0.261	0.094	0.124	0.284

below a theoretically possible value even with the normal tissues. Since a value of less than 1 mole per mole of tyrosine may be assumed unlikely, one may suppose the reaction responsible to be much slower than the one involving oxygen consumption. Or, on the other hand, carbon dioxide

production may be concomitant with later stages of tyrosine oxidation rather than with the stage involving the 1st atom utilized. The latter explanation is more in keeping with the results obtained by Felix and Zorn (8) in their analysis of tyrosine oxidation by minced liver tissue.

When kidney tissue is examined under the same conditions, comparison of the Q_{O_2} values obtained with tyrosine and in the absence of the substrate (Table IV) confirms the previously described ability of this tissue to oxidize the amino acid. The presence of the substrate thus causes an average increased oxygen consumption of 14.4 per cent in the three groups of animals receiving vitamin C. This increment is to be contrasted with that of 90.5

TABLE IV

Tyrosine Oxidation by Kidney Slices

The results obtained with four guinea pigs are represented in each group.

	Normal	Scorbutic	Ascorbic acid injected	
			Scorbutic	Normal
Control flasks, without substrate				
Tissue dry weight, average,				
mg....	8.4	6.8	6.5	7.9
Q_{O_2}, range	6.75 – 9.21	6.20–8.63	8.00 – 9 22	9.10 – 9.59
" average. .	8.09	7.82	8.87	9.23
With 5.52 micromoles tyrosine				
Tissue dry weight, average,				
mg..	8.0	7.1	6.6	7.7
Q_{O_2}, range	7.84 –10.94	7.02–8.59	8.51 –11.66	10.05 –10.84
" average. .	9.18	7.85	10.40	10.60
$\dfrac{O}{\text{Tyrosine}}$, range	0.032– 0.746	0 –0.130	0.146– 0.616	0.390– 0.616
" average.. ..	0.420	0.084	0.462	0.490

per cent exhibited by the liver tissue with the same three groups of guinea pigs. That the kidney slices oxidize tyrosine less readily than do liver slices is likewise to be seen in the low ratios of oxygen to tyrosine, less than 0.5 atom of extra oxygen being utilized for each mole of tyrosine present. It is, however, evident from Table IV that even though the kidney lacks the ability to oxidize tyrosine to the same extent as does the liver, what ability it does possess in this direction is almost completely lost as the animal becomes scorbutic. Carbon dioxide production, as is evident in Table V, reaches only insignificant proportions in the process of tyrosine metabolism by kidney tissues under the conditions of these experiments.

With the information from the entire group of experiments, it is obvious that the liver tissue exhibits the greater oxidation of the substrate, tyrosine. Since the same tissue when deficient in vitamin C loses this property, it must be determined whether or not the addition of the vitamin to the reaction

TABLE V

Carbon Dioxide Production by Kidney Slices

The results obtained with four guinea pigs are represented in each group.

		Normal	Scorbutic	Ascorbic acid injection	
				Scorbutic	Normal
Control flasks,	Q_{CO_2}, range	5.30–8.40	5.75–6.64	6.01–6.23	6.12–7.61
without substrate	" average	6.39	6.24	6.13	6.93
With 5.52 micro-	" range	6.00–8.44	5.22–7.50	5.26–6.63	6.54–7.81
moles tyrosine	" average	7.03	6.25	6.28	7.04
	$\dfrac{CO_2}{Tyrosine}$, range	0 –0.227	0 –0.211	0 –0.097	0 –0.097
	" aver- age	0.129	0.032	0.045	0.030

TABLE VI

Tyrosine Oxidation

Liver slices with 0.3 mg. of extra ascorbic acid.

	Control	With tyrosine	
	Q_{O_2}	Q_{O_2}	$\dfrac{O}{Tyrosine}$
Scorbutic animals	2.23	4.23	1.64
	2.95	3.56	0.53
	2.31	3.01	0.66
Average	2.50	3.60	0.943
Normal animals	2.06	3.71	1.25
	2.09	3.33,	0.78
	2.20	3.37	0.78
	2.28	3.33	0.78
Average	2.16	3.44	0.898

mixture will cause the liver to oxidize tyrosine again. Experiments with scorbutic and normal guinea pigs were carried out in order that this point might be tested.   0.3 mg. of crystalline *l*-ascorbic acid was dissolved in the Ringer-phosphate solution in the side arm.   In some of the flasks, 1.0 mg.

of *l*-tyrosine was also included. By including the vitamin in those flasks containing no substrate as well as in the experimental ones any oxygen consumed in the oxidation of ascorbic acid may be considered the same in both groups. The oxygen consumed in the oxidation of tyrosine is then calculated as in the other experiments by deducting that of the control flasks. A summary of the results is presented in Table VI. The oxygen to tyrosine ratio of 0.94 clearly indicates that the addition of ascorbic acid to the scorbutic tissue *in vitro* has restored the ability of the tissue to oxidize the amino acid. The ratio, 0.90, obtained when the vitamin is added to normal tissue corresponds to the ratio, 1.14 (Table II), obtained with normal tissue containing no added ascorbic acid. Carbon dioxide production parallels that of the oxygen consumption but as in the previous experiments the calculated ratios are somewhat less.

DISCUSSION

The results of these experiments show that the ability of slices of surviving liver tissue from guinea pigs to oxidize the amino acid, tyrosine, is dependent upon a normal intake of ascorbic acid, for vitamin C-deficient liver exhibits almost no oxidation of the compound. That the property is not permanently lost has been shown by the fact that scorbutic animals when administered the vitamin are then able to oxidize tyrosine. Likewise the addition of the vitamin to scorbutic tissue *in vitro* results in the subsequent return of the normal function.

With the finding that guinea pig liver slices oxidize tyrosine only in the presence of an adequate amount of ascorbic acid, it would appear that the vitamin is a necessary and key component of the enzyme systems responsible for the oxidation of the amino acid. It may even be the prosthetic group of one of the enzymes acting as a respiratory catalyst in tyrosine metabolism. The attractiveness of the hypothesis does not, however, overcome the lack of conclusive experimental evidence. Even though the *in vitro* and *in vivo* experiments point to a catalytic rôle and a demonstrably specific one in the latter case (2), an indirect action of ascorbic acid in tyrosine oxidation must also be considered.

The *in vitro* oxidation of tyrosine by liver slices is in agreement with results previously reported from other laboratories (9). However, the observed ratio of 1 atom of oxygen for each mole of tyrosine present is only one-fourth of that found by Bernheim and Bernheim (10, 11) and by Felix and Zorn (8) when liver brei was used. The lower ratio in the case of the slices should not be interpreted as indicating a different mechanism of oxidation from that of brei, for more extensive investigation has shown that the higher ratios are dependent upon the use of a greater proportion of liver tissue

per mg. of tyrosine.[1] Likewise, it is to be observed from the left-hand graph of Fig. 1 that the oxygen consumption of neither the basal nor experimental flasks had reached a maximum at the end of 3 hours. Continuation of the experiment would undoubtedly have yielded a higher ratio.

Previously described results relating tyrosine metabolism in the intact animal to the ascorbic acid intake may be correlated with these results, demonstrating that the oxidation observed is dependent upon a normal state of vitamin C nutrition. It may be assumed that the normal catabolic breakdown of tyrosine occurs readily only when the liver contains an adequate amount of the vitamin. When the vitamin is present in inadequate amount, the extra tyrosine fed to the intact animal does not proceed through its customary channels of catabolism, but instead is excreted principally as p-hydroxyphenylpyruvic acid and other partial metabolites. From this it may be concluded that the oxidative deamination of tyrosine occurs entirely independently of the state of vitamin C saturation.

The results obtained with the feeding of extra l-phenylalanine also become more understandable. Since this amino acid when fed to the scorbutic guinea pig or infant (3, 4) likewise yields principally the tyrosine keto acid, the results of the in vitro experiments lead to the conclusion that the failure of phenylalanine to be metabolized occurs after its conversion to tyrosine. Such a conclusion is even more tenable in view of the demonstration by Moss and Schoenheimer (12) of the readiness with which phenylalanine is converted to tyrosine.

It is also of interest to point out at this time that the results of the in vivo and in vitro experiments clearly lead to definite suggestions pertaining to other phases of the metabolism of phenylalanine and tyrosine. Since the greater portion of either compound is not oxidized in the absence of an adequate amount of ascorbic acid, it is obvious that the full caloric value of either is not available. It is likewise evident that the specific dynamic action of these two amino acids must be greatly reduced when the ascorbic acid intake is low. And finally, they must also be less ketogenic in the scorbutic than in the normal animal.

SUMMARY

1. With l-tyrosine present as substrate the Q_{O_2} values of surviving liver slices from normal guinea pigs are markedly increased above those of control slices. From the increased Q_{O_2} a ratio of 1 atom of extra oxygen per mole of tyrosine present may be calculated.

2. Liver slices from scorbutic guinea pigs, on the other hand, do not exhibit the increased oxygen consumption in the presence of tyrosine.

[1] Sealock and Goodland, unpublished data.

3. The scorbutic liver may regain its ability to oxidize the amino acid, for the administration of ascorbic acid either *in vivo* or *in vitro* results in increased Q_{O_2} values in the presence of tyrosine.

4. With kidney slices, the oxidation of tyrosine is also dependent upon an adequate ascorbic acid level, although the importance of the kidney in tyrosine catabolism appears definitely less than that of the liver.

5. Carbon dioxide production, in general, parallels oxygen consumption, although the observed differences are of smaller magnitude.

6. The oxidation of tyrosine by normal and lack of oxidation by scorbutic tissues has been correlated with *in vivo* results obtained previously with normal and scorbutic guinea pigs.

BIBLIOGRAPHY

1. Sealock, R. R., *Federation Proc.*, **1**, 287 (1942).
2. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135**, 251 (1940).
3. Sealock, R. R., Perkinson, J. D , Jr., and Basinski, D. H., *J. Biol. Chem.*, **140**, 153 (1941).
4. Levine, S Z , Gordon, H. H., and Marples, E , *J. Clin. Invest.*, **20**, 209 (1941).
5. Marsh, M. E , *J. Nutr.*, **13**, 109 (1937).
6. Stotz, E., Harrer, C. J., Schultze, M. O., and King, C. G., *J. Biol. Chem.*, **120**, 129 (1937).
7. Laser, H., *Biochem J* , **36**, 319 (1942).
8. Felix, K., and Zorn, K , *Z. physiol. Chem.*, **268**, 257 (1941).
9. Kisch, B., *Biochem. Z.*, **242**, 26 (1931)
10. Bernheim, F., and Bernheim M. L. C., *J. Biol. Chem.*, **107**, 275 (1934).
11. Bernheim, F., *J. Biol. Chem.*, **111**, 217 (1935).
12. Moss, A. R., and Schoenheimer, R., *J. Biol. Chem* , **135**, 415 (1940).

ON BACTERIAL GLYCOGEN: THE ISOLATION FROM AVIAN TUBERCLE BACILLI OF A POLYGLUCOSAN OF VERY HIGH PARTICLE WEIGHT *

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Glycogen is an almost ubiquitous constituent of the animal cell, but in plants its occurrence appears to be limited to the primitive thallophytes A polysaccharide resembling animal glycogen in many respects has, however, been isolated from sweet corn (1-3) The presence of glycogen in bacteria has often been claimed on the basis of the color reaction with iodine, but only rarely substantiated by the isolation of the pure polysaccharide, and even when this was done, the final identification usually rested on the isolation of glucose phenylosazone and was inconclusive, as this derivative could, of course, equally well have originated from mannose or fructose

The older literature contains several references to the occurrence of glycogen in tubercle bacilli (4) Laidlaw and Dudley (5) reported the isolation of small amounts of glycogen (0 33 gm from 1000 gm of wet bacilli) from tubercle bacilli of the human type that had been grown on broth and treated with chloroform-alcohol previous to the extraction of the polysaccharides with alkali They characterized the compound by the iodine reaction, rotation following hydrolysis, and isolation of glucosazone The more recent literature dealing with the specific polysaccharides of tubercle bacilli likewise frequently mentions the presence of contaminating substances, giving the iodine reaction for glycogen, which were removed either by fractionation with alcohol (6) or by digestion with salivary amylase (7, 8)

The observation that buffered aqueous extracts of tubercle bacilli of the avian type, when subjected to high speed centrifugation, deposited considerable amounts of a macro molecular polysaccharide resembling glycogen offered an opportunity for a more detailed study of glycogen derived from a pathogenic microorganism The purpose of this work, in addition to establishing beyond doubt the exclusive presence of glucose in this polysaccharide, was to gain an insight into the molecular size of the compound.

The starting material consisted of organisms that had been grown in the synthetic Sauton medium   The extraction of the disintegrated bacilli with borate buffer of pH 8 4 yielded opalescent solutions which by centrifugation at 31,000 g could be separated into the sedimentable glycogen fraction and a supernatant containing a nucleoprotein   This compound will form the subject of a later communication   When dilute trichloroacetic acid was employed as extracting fluid, no nucleoprotein was encountered in the extract, the high speed supernatant and the sediment both appeared to contain polysaccharides exclusively   The preparation obtained as the high speed sediment of the trichloroacetic acid extract exhibited particularly sharp boundaries in the ultracentrifuge and was used for the determination of the particle weight of the polysaccharide. (See Fig 1)

It is peculiar that none of the specific polysaccharides of the avian tubercle bacillus (9) was encountered in the fractions examined   This
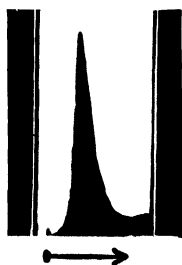
FIG 1 Ultracentrifuge pattern of bacterial glycogen (1 per cent solution in barbiturate buffer, pH 7 8) after 840 seconds in a centrifugal field of 39,000 g

must be due to a particular availability of the bacterial glycogen to extraction under the conditions employed and also, although this would not explain the absence of the specific polysaccharides from the high speed supernatants, to the low molecular weight (9) of the specific substances The amount of glycogen isolated from the bacteria was considerable, when small lots were worked up, yields of almost 4 per cent were obtained.

The bacterial glycogen fraction was characterized by the specific rotation of its hydrolysis product, which agreed perfectly with the rotation of d-glucose, and by the isolation of two hydrazone derivatives of glucose in the pure state, viz , the p-nitrophenylhydrazone and the p-bromophenylhydrazone of glucose.

The physical properties of one glycogen specimen were studied in greater detail.   The apparent partial specific volume of this preparation was $V_{27} = 0 63$ (in water), quite similar to the value of 0 65 found for rabbit liver glycogen (10)   A particle weight of 12 1 million was calculated from

sedimentation velocity (s_{20} = 170 Svedberg units) and diffusion (D_{20} = 0.925 × 10^{-7}). The frictional ratio f/f_0 = 1.59 corresponds to an axial ratio of about 11 for an elongated ellipsoid. The specific viscosity of a 1 per cent solution in water was found at 0.09, which is in very good agreement with values reported in the literature (11–13). The evaluation of the viscosity and diffusion data led to a particle weight of 13.2 million. It is evident that the shape of the glycogen molecule deviates somewhat from the spherical form. (Compare the discussion in (10, 12, 13).)

It has long been clear that glycogen, as isolated from the animal cell, represents a family of polyglucosans of different degrees of polymerization. The differences in solubility observed by Willstätter and Rohdewald (14) for lyo- and desmoglycogen are probably attributable to varying molecular sizes (12). Estimates of molecular dimensions, based on electron microscopic observations, show considerable divergences (15, 16). That at least a fraction of animal glycogen occurs in a particulate form has been observed repeatedly (17, 18). It was fortunate that some of the glycogen preparations from tubercle bacilli were found monodisperse and could be examined in the analytical ultracentrifuge. Rabbit liver glycogen appears to exhibit a much greater spread in particle size (10, 19). It is probable that the particle weight found for the bacterial preparations, although the highest reported so far, does not represent the upper limit for native glycogen.

It is noteworthy that the occurrence of glucose in tubercle bacilli appears limited to two groups of compounds; viz., the bacterial glycogen discussed here and the acetone-soluble fat in which it is present as trehalose (20). It may also be mentioned that the glycogen preparations from tubercle bacilli were free of nitrogen and phosphorus. The determination of the branch structure (21) of the bacterial glycogen was unfortunately precluded by the, at least temporary, difficulty of securing sufficient material.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Preparation of Glycogen*</div>

Starting Material[1]—Tubercle bacilli of the avian strain (obtained through the courtesy of Dr. M. M. Steinbach) were grown in the synthetic medium according to Sauton (22) at pH 7.4 and 38°. The 6 week-old cultures were filtered off with suction, thoroughly washed with distilled water, suspended in a small amount of water, and dried in the frozen state in a vacuum. The dried organisms, which were acid-fast and showed the normal microscopic picture, contained 4.7 per cent N. The yield in dry bacilli amounted to about 18 gm. per liter of medium.

[1] We are highly indebted to Dr. W. R. Kessler for valuable aid in the cultivation of the microorganisms.

The microorganisms were, previous to the extraction of water-soluble substances, treated with ice-cold ether (20 cc. per gm. of bacilli) for 24 hours. This treatment removed 4 to 5 per cent of lipid material; *i.e.*, roughly one-third of the bacillary lipids (20).

Extraction with Borate Buffer—In a typical experiment, 1.00 gm. of tubercle bacilli was mixed with 5 gm. of washed, very fine Pyrex glass powder (diameter 3 μ) and a small amount of borate buffer of pH 8.5, and the stiff paste was ground in a mortar for 30 minutes. The microscopic observation of the preparation at this stage revealed the presence of acid-fast rods with less well marked contours together with a high proportion of acid-fast fragments. After the addition of 70 cc. of the borate buffer, the mixture was kept in the refrigerator overnight and then spun in a refrigerated angle centrifuge at 4000 R.P.M. (1900 *g*). The opalescent supernatant was subjected to a preliminary centrifugation at 8000 R.P.M. (5000 *g*) for 30 minutes and then centrifuged for 3 hours at 20,000 R.P.M. (31,000 *g*). A refrigerated International centrifuge with multispeed attachment was used. This treatment resulted in the separation of a colorless translucent jelly from a clear protein-containing supernatant. The jelly was dissolved in borate buffer of pH 8.5 and the strongly opalescent solution subjected to two more cycles of centrifugation at intermediate and high speeds. At this stage, no protein could be detected by means of trichloroacetic acid in either the sediment or the supernatant. The transparent sediment was dissolved in distilled water and the solution dialyzed against running tap water for 24 hours and against ice-cold distilled water for 48 hours. Following the evaporation of the water from the frozen solution in a vacuum, 38.7 mg. of *glycogen* (3.9 per cent of the starting material) were obtained as a voluminous white felt. The yields of this fraction varied between 2.5 and 4 per cent in inverse proportion to the size of the lot processed in one operation.

The protein-containing supernatant from the high speed sedimentation was electrophoretically homogeneous. The examination in the Tiselius cell revealed the presence of one component migrating as a sharp boundary with a mobility (in 0.02 M barbiturate buffer of pH 7.74) of -7.2×10^{-5} sq. cm. per volt per second (descending boundary). The removal of the water from the frozen dialyzed solution in a vacuum yielded 34.0 mg. of a *nucleoprotein* (3.4 per cent of the starting material) containing N 9.1, P 0.93. This material gave strong color reactions for desoxyribose nucleic acid with diphenylamine (23) and cysteine (24) and exhibited an absorption maximum in the ultraviolet at 2590 A.

Extraction with Trichloroacetic Acid—A suspension of 20.0 gm. of tubercle bacilli in 140 cc. of water was ground with 50 gm. of washed Pyrex glass

powder, 140 cc. of 0.2 N trichloroacetic acid in water were added, and the mixture was shaken mechanically for 17 hours at 0°. The bacterial residue was removed by centrifugation and washed with 0.1 N trichloroacetic acid. The combined supernatants were dialyzed against running tap water for 64 hours and, following concentration by pervaporation to a volume of about 70 cc., against ice-cold distilled water for 16 hours. This solution was subjected to several cycles of fractional high speed centrifugation, as described in the preceding section. From the supernatant, Fraction Tr-1 was obtained by evaporation of the water in the frozen state in a vacuum as 162.2 mg. of a faintly yellowish powder. The sediment, treated in the same manner, yielded the white glycogen, Fraction Tr-2, weighing 101.9 mg. This material served for the study of the physical properties to be described later in the paper.

Chemical Properties—The glycogen fractions obtained by the centrifugation at high speed of the borate or trichloroacetic acid extracts of the tubercle bacilli formed snow-white powders that were free of nitrogen and phosphorus. They were easily soluble in water, giving solutions too opalescent to permit the determination of their optical rotation. The Molisch reaction was positive; the orcinol reactions for pentoses according to Bial and to Neumann, the keto sugar reaction of Seliwanoff and Weehuizen, and the uronic acid reaction of Tollens and Neuberg were all negative (25). For the quantitative determination of reducing sugars according to Hagedorn and Jensen (26) the samples were heated at 100° with 1 N hydrochloric acid (27). Hydrolysis was complete at the end of 2 hours. The values for total reducing sugars (calculated as glucose) lay for different samples between 102 and 108 per cent. Without hydrolysis, the preparations showed no reducing properties.

The reddish brown color, produced by the addition of 1 drop of a 0.33 per cent iodine solution in 0.67 per cent potassium iodide (saturated with sodium chloride) to 1 drop of a concentrated solution of the bacterial glycogen, was not much different from that obtained with purified liver glycogen. Some differences were, however, observed when the procedure recently described by Sumner and Somers (3) was followed. The addition of 3 drops of a 0.005 N iodine solution in potassium iodide to 10 cc. of a 0.25 per cent solution of the polysaccharide (half saturated with ammonium sulfate) conferred a light red-brown color on the solution of liver glycogen, whereas the bacterial glycogen solution acquired a pure yellow color that was much more intense than that of a control solution from which the polysaccharide was omitted. This was particularly noticeable when the iodine drops were observed before dispersing in the solution.

The preparations discussed here lacked specific activity and gave no

precipitin tests, when examined with rabbit antisera against the tubercle bacillus strain used in these experiments and also with pooled anti-avian rabbit sera obtained through the courtesy of Dr. M. Heidelberger.[2]

The electrophoretic behavior of the bacterial glycogen preparations will be discussed later in the paper together with their other physical properties; but it may be mentioned here that Fraction Tr-1, isolated from the high speed supernatant of the trichloroacetic acid extract, consisted, in contrast to the sedimentable macro molecular glycogen fractions, of three electrophoretic components with the following mobilities and relative proportions (barbiturate buffer of pH 7.8, descending boundaries): I, -0.6 (24 per cent); II, -1.2 (63 per cent); III, -2.5 (13 per cent) $\times 10^{-5}$ sq. cm. per volt per second.

Enzymatic Hydrolysis—The action of amylase on the bacterial glycogen was examined by means of an *Aspergillus* amylase preparation, supplied by the Wallerstein Laboratories, New York. The experiments were carried out at 37° in $M/15$ phosphate buffer (containing 0.5 per cent of sodium chloride) of pH 6.2. The action of the enzyme proceeded at a rather slow rate. After 40 minutes 12.6 per cent of reducing sugars (calculated as glucose) was liberated; after 80 minutes, 20.6 per cent; after 120 minutes, 22.6 per cent. From purified liver glycogen, the following amounts of reducing sugars (calculated as glucose) were set free under identical conditions: after 40 minutes, 27.6 per cent; after 80 minutes, 34.4 per cent; after 120 minutes, 38.2 per cent.

Identification of Glucose

Hydrolysis—A solution of 400 mg. of a bacterial glycogen preparation (obtained from a borate buffer extract) in 40 cc. of 1 N sulfuric acid was boiled with a reflux for 3 hours. The addition of a warm barium hydroxide solution to the chilled hydrolysate until it was slightly alkaline was followed by acidification with dilute sulfuric acid and complete neutralization with a small amount of barium carbonate. The neutral filtrate was concentrated *in vacuo* to a small volume and then, by the addition of water, adjusted to a volume of exactly 100 cc. This solution was, by the Hanes modification of the Hagedorn-Jensen method (28), found to contain a total of *365 mg. of reducing sugars (as glucose)*, whereas from the optical rotation of the solution ($\alpha_D^{25} = +0.38°$) a *d-glucose content of 362 mg.* was calculated.

Glucose p-Nitrophenylhydrazone—A portion of the sugar solution, containing 175 mg. of glucose by analysis, was concentrated to dryness *in vacuo* and the remaining sirup heated with a reflux with 200 mg. of freshly recrystallized p-nitrophenylhydrazine and 3 cc. of methyl alcohol. The clear solution was evaporated *in vacuo* and the residue extracted with small

[2] We are very grateful to Miss Adele Karp for these experiments.

portions of ice-cold methyl alcohol, which left behind 89.3 mg. of the crude hydrazone melting at 185°. An additional crop, weighing 31.2 mg., was obtained from the chilled concentrated mother liquor. Two recrystallizations from 98 per cent alcohol yielded 55.2 mg. of pure *d-glucose p-nitrophenylhydrazone* (29) as glistening orange prisms melting (with decomposition) at 190–191°. The melting point of a mixture of this compound with an authentic specimen (m.p. 190–190.5°) prepared from pure *d*-glucose (Bureau of Standards) was at 189°. The substance was, for analysis, dried at 61° over P_2O_5 *in vacuo*.

$C_{12}H_{17}N_3O_7$ (315.3). Calculated. C 45.7, H 5.4, N 13.3
Found. " 45.8, " 5.5, " (Dumas) 13.1

The mother liquors from the recrystallizations yielded 26.4 mg. of an almost pure hydrazone.

Glucose p-Bromophenylhydrazone—Another portion of the sugar solution, containing 180 mg. of glucose by analysis, was evaporated to dryness *in vacuo*. The residue was heated with a reflux with 200 mg. of freshly recrystallized *p*-bromophenylhydrazine and 5 cc. of 80 per cent alcohol for 30 minutes and the solution evaporated to dryness *in vacuo*. The addition of 1.5 cc. of warm chloroform to the solution of the residue in 1 cc. of warm absolute alcohol produced the deposition of crystals from the chilled mixture. Two more recrystallizations from alcohol-chloroform (2:3) yielded 33.0 mg. of the pure *d-glucose p-bromophenylhydrazone* ((25) p. 158) as white hexagonal crystals which melted at 146–147°. The combined mother liquors yielded 72.8 mg. of the same substance. No depression of the melting point was observed on admixture of an authentic specimen of the hydrazone. For analysis, the compound was dried *in vacuo* over P_2O_5 at 61°.

$C_{12}H_{17}BrN_2O_5$ (349.2). Calculated. C 41.3, H 4.9, N 8.0
Found. " 41.0, " 5.0, " (Dumas) 7.8

Physical Properties

The studies presented in this section were carried out with the bacterial glycogen preparation, Fraction Tr-2, isolated as the high speed sediment from the trichloroacetic acid extract and purified in the manner described before.

Electrophoretic Mobility—The electrophoretic properties of the bacterial glycogen were examined in the Tiselius cell at 1.5°, with the optical arrangement of Longsworth. The mobility of the single component was low. In 0.02 M barbiturate buffer of pH 7.8 it was found at −0.96 (descending) and −0.97 (ascending) $\times 10^{-5}$ sq. cm. per volt per second. Other preparations had even lower mobilities; *e.g.*, −0.6.

Ultracentrifuge Studies—The sedimentation velocity of the preparation (1 per cent solution in barbiturate buffer of pH 7.8) was examined in an air-driven vacuum ultracentrifuge.[3] A typical pattern obtained with this preparation is reproduced in Fig. 1. It may be seen that the material sedimented homogeneously with the exception of a small amount of some very heavy polydisperse material. The sedimentation constant of the bacterial glycogen was $s_{20} = 170$ Svedberg units.

Diffusion—The diffusion constant (1 per cent solution in barbiturate buffer of pH 7.8), determined in the Tiselius apparatus at 1.5°, was $D_{20} = 0.925 \times 10^{-7}$. The solutions used for the study of sedimentation and diffusion were, previous to the determinations, dialyzed against a large volume of barbiturate buffer in the refrigerator.

Partial Specific Volume—The preparation, recovered from its aqueous solution by evaporation in the frozen state in a vacuum, was, previous to this determination, dried to constant weight *in vacuo* over P_2O_5. The partial specific volume of the glycogen (0.5 per cent solution in distilled water) was determined at 27° by means of the falling drop method.[4] It was found as $V_{27} = 0.63$.

Viscosity—The specific viscosity of a 1.04 per cent solution in distilled water at 20.2°, determined in an Ostwald viscosimeter, was $\eta_{sp} = 0.09$.

Particle Weight and Shape—The particle weight of the preparation of bacterial glycogen, based on the sedimentation and diffusion constants and the partial specific volume (30) was calculated as 12.1 million. The frictional ratio, computed from the same values, $f/f_0 = 1.59$, corresponds to an axial ratio of about 11 for an elongated ellipsoid (30). The viscosity increment, calculated from the specific viscosity and the partial specific volume of the glycogen preparation (31), agrees with an axial ratio of about 10 for an elongated molecule. This leads to a particle weight of 13.2 million (from viscosity and diffusion).

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SUMMARY

Glycogen preparations of very high particle weight were obtained from avian tubercle bacilli by the sedimentation at high speed of borate buffer

or trichloroacetic acid extracts.   d-Glucose was demonstrated as the sole product of the hydrolysis of the polysaccharide.   A particle weight of 12.1 million was calculated from the rate of sedimentation (s_{20} = 170 Svedberg units), diffusion (D_{20} = 0.925 × 10^{-7}), and partial specific volume (V_{27} = 0.63).   Computations based on the specific viscosity led to a particle weight of 13.2 million.   The isolation of a nucleoprotein fraction is mentioned.

BIBLIOGRAPHY

1. Morris, D. L., and Morris, C. T., J. Biol. Chem., **130,** 535 (1939).
2. Hassid, W. Z., and McCready, R. M., J. Am. Chem. Soc., **63,** 1632 (1941).
3. Sumner, J. B., and Somers, G. F., Arch. Biochem., **4,** 7 (1944).
4. Wells, H. G., and Long, E. R., The chemistry of tuberculosis, Baltimore, 2nd edition, 79 (1932).
5. Laidlaw, P. P., and Dudley, H. W., Brit. J. Exp. Path., **6,** 197 (1925).
6. Gough, G. A. C., Biochem. J., **26,** 248 (1932).
7. Heidelberger, M., and Menzel, A. E. O., J. Biol. Chem., **118,** 79 (1937).
8. Menzel, A. E. O., and Heidelberger, M., J. Biol. Chem., **127,** 221 (1939).
9. Karjala, S. A., and Heidelberger, M., J. Biol. Chem., **137,** 189 (1941).
10. Bridgman, W. B., J. Am. Chem. Soc., **64,** 2349 (1942).
11. Staudinger, H., and Husemann, E., Ann. Chem., **530,** 1 (1937).
12. Meyer, K. H., and Jeanloz, R., Helv. chim. acta, **26,** 1784 (1943).
13. Meyer, K. H., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3,** 109 (1943).
14. Willstätter, R., and Rohdewald, M., Z. physiol. Chem., **225,** 103 (1934).
15. Husemann, E., and Ruska, H., J. prakt. Chem., **156,** 1 (1940).
16. von Ardenne, M., Z. phys. Chem., Abt. A, **187,** 1 (1940).
17. Lazarow, A., Science, **95,** 49 (1942).
18. Loring, H. S , and Pierce, J. G., J. Biol. Chem., **148,** 35 (1943).
19. Mystkowski, E. M., Biochem. J., **31,** 716 (1937).
20. Anderson, R. J., in Zechmeister, L., Fortschritte der Chemie organischer Naturstoffe, Vienna, **3,** 145 (1939).
21. Haworth, W. N., Hirst, E. L., and Isherwood, F. A., J. Chem. Soc., 577 (1937).
22. Sauton, B., Compt. rend. Acad. Sc., **155,** 860 (1912).
23. Dische, Z., Mikrochemie, **8,** 4 (1930).
24. Dische, Z., Proc. Soc. Exp. Biol. and Med., **55,** 217 (1944).
25. van der Haar, A. W., Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin (1920).
26. Hagedorn, H. C., and Jensen, B. N., Biochem. Z., **135,** 46 (1923); **137,** 92 (1923).
27. Sahyun, M., and Alsberg, C. L., J. Biol. Chem., **93,** 235 (1931).
28. Hanes, C. S., Biochem. J., **23,** 99 (1929).
29. Alberda van Ekenstein, W., and Blanksma, J. J., Rec. trav. chim. Pays-Bas, **22,** 434 (1903).
30. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
31. Mehl, J. W , Oncley, J. L., and Simha, R., Science, **92,** 132 (1940).

THE CITRATE CONTENT OF THE SKELETON AS INFLUENCED BY PROLONGED FEEDING OF ACID-PRODUCING AND BASE-PRODUCING SALT*

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It has been repeatedly demonstrated that the soft tissues of the body do not contain appreciable stores of citrate (1, 2). Recently, however, Dickens (3) showed that the concentration of citrate in bone is many times higher than that of other body tissues and this suggested the possibility of a considerable reserve of citrate in the body. Class and Smith (4) have shown that this store of citrate was not the origin of the increased urinary excretion of citrate following the administration of sodium bicarbonate or sodium malate. The present investigation was designed to determine whether or not the bone citrate may be augmented or mobilized by changes in acid-base balance and by the administration of large quantities of citrate.

Methods

Thirty young albino rats were divided into three equal groups and put on a diet of commercial dog chow. One group received a supplement of 2 gm. of sodium citrate per kilo of body weight per day. The other groups received equivalent amounts of ammonium chloride and sodium chloride respectively, the animals given sodium chloride serving as controls. The concentrations of the salt solutions were adjusted so that each rat received 2 ml. of the solution per 100 gm. of body weight twice daily. The exact concentrations were as follows: sodium citrate 5 gm. of $Na_3C_6H_5O_7 \cdot 2H_2O$ per 100 ml., ammonium chloride 2.8 gm. of NH_4Cl per 100 ml., and sodium chloride 3.0 gm. of $NaCl$ per 100 ml. The salts were administered twice daily by stomach tube for 65 days, during which time the animals were observed and weighed regularly. At the end of this period the animals were sacrificed by decapitation. The femurs were dissected away from the soft tissues, dried at 105° for 48 hours, weighed, and each femur dissolved separately in 10 per cent trichloroacetic acid solution. An aliquot of this solution was analyzed for citric acid by the method of Pucher, Sherman, and Vickery (5). The calcium content of the bone was determined by the method of Tisdall and Kramer (6) applied to an ashed aliquot of the tri-

chloroacetic acid filtrate. Phosphorus was determined by the method of Fiske and Subbarow (7).

Several days before the animals were sacrificed a sample of 0.2 ml. of blood was obtained from the tail and the blood urea determined by the colorimetric method of Ormsby (8). A larger sample of blood was collected from some of the rats at the time they were sacrificed and the blood urea checked by the usual urease-aeration technique (9). No significant differences were found in the results obtained by the two methods.

DISCUSSION

The average gain in weight of each group of rats after 65 days of supplementation with sodium citrate, ammonium chloride, and sodium chloride is given in Table I. It is readily apparent that there was no significant difference in the growth rate of the three groups. The appearance and activity of all the rats were also comparable. These observations indicate that the prolonged oral administration of relatively large amounts of sodium citrate, which is metabolized to give alkaline end-products, or ammonium chloride, which is metabolized to give acid end-products, had very little influence on the nutritive condition of the animals. Since the control animals in this series received sodium chloride solution by stomach tube, their growth rate was compared with another group of animals on the same diet but receiving no supplements. No significant differences were found. In order to ascertain whether any renal damage had been produced in the animals as a result of alkali or acid administration the blood urea nitrogen and the kidney weights were studied. The average results which are given in Table I when analyzed statistically show no differences in the three groups of animals and do not support the contention that the kidney function may be impaired as a result of repeated administration of base-producing salt. Nor does the administration of acid-producing ammonium chloride have any adverse effects on renal function. These results substantiate the findings of Free, Davies, Gustafson, and Myers (10) and Kirsner, Palmer, and Humphreys (11) who found that alkali administration did not alter renal function. The results on the composition of the bones, which are given in Table II, are the average of separate analyses of the two femurs on each of the ten rats in the group. The fact that the citrate content of the leg bones of the three groups of animals did not differ significantly is a definite indication that the acid-base changes or the administration of large amounts of citrate has very little effect on the citrate content of the skeleton. These results are in accord with those of Class and Smith (4) who noted that the stimulation of citrate excretion by administration of sodium bicarbonate or sodium malate did not cause any significant alteration in skeletal citrate.

Values for the calcium and phosphorus content of the femurs are presented in Table II. It will be seen that there is no significant variation in the calcium or phosphorus content of the bones of the three groups of animals.

The results of this study indicate that the rat can readily resist a strain on the acid-base equilibrium produced by either the administration of an acid-producing or a base-producing salt. The principal criteria for this

TABLE I

Nutritive Condition and Kidney Function

Group	Weight gain	Average kidney weight	Blood urea nitrogen
	gm.	*gm. per 100 gm.*	*mg. per 100 cc.*
Sodium citrate	146 ± 40*	0.43 ± 0.03	37 ± 6
Ammonium chloride	133 ± 25	0.43 ± 0.03	40 ± 8
Sodium chloride	138 ± 27	0.42 ± 0.03	37 ± 9

* Standard deviation.

TABLE II

Acid-Base Changes and Composition of Skeleton

Group	Composition of bone, gm. per 100 gm dry basis		
	Citrate	Calcium	Phosphorus
Sodium citrate	0.72 ± 0.08*	19.3 ± 3.0	11.1 ± 0.9
Ammonium chloride.. ...	0.69 ± 0.07	20.4 ± 4.0	12.7 ± 2.7
Sodium chloride	0.67 ± 0.07	19 5 ± 2.8	11.2 ± 0.8

* Standard deviation.

are the unaltered growth rate and the absence of any harmful effect on the kidneys.

SUMMARY

Three groups of rats were respectively supplemented with sodium citrate, ammonium chloride, and sodium chloride administered by stomach tube.

The oral administration of sodium citrate or ammonium chloride in relatively large amounts for 65 days had no influence on the nutritive condition of the animals as evidenced by appearance and growth rate. Blood urea studies did not indicate any impairment in kidney function.

The citrate content of the skeleton was not altered by the prolonged feeding of sodium citrate or ammonium chloride.

BIBLIOGRAPHY

1. Ostberg, O., *Skand. Arch. Physiol.*, **62**, 81 (1931).
2. Smith, A. H., and Orten, J. M., *J. Nutr.*, **13**, 601 (1937).
3. Dickens, F., *Biochem. J.*, **35**, 1011 (1941).
4. Class, R. N., and Smith, A. H., *J. Biol. Chem.*, **151**, 363 (1943).
5. Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, **113**, 235 (1936).
6. Tisdall, F. F., and Kramer, B., *J. Biol. Chem.*, **48**, 1 (1921).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Ormsby, A. A., *J. Biol. Chem.*, **146**, 595 (1942).
9. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **24**, 117 (1916).
10. Free, A. H., Davies, D. F., Gustafson, G. E., and Myers, V. C., *Federation Proc.*, **1**, pt. 2, 110 (1942).
11. Kirsner, J. B., Palmer, W. L., and Humphreys, E. M., *Arch. Path.*, **35**, 207 (1943).

THE DETERMINATION OF FREE CHOLINE
IN ANIMAL TISSUES

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The physiological importance of choline is well recognized. Its two primary functions as a donor of methyl groups and as a constituent of lecithin and sphingomyelin have received considerable attention. Virtually nothing is known about the relatively small but probably significant amounts of free or unbound choline occurring in body tissues. Suitable methods for determining free choline in animal tissues have not heretofore been available. An adaptation to body tissues of the microbiological method for measuring free choline in plasma and urine (1) appeared to offer the best possibilities. The development of such a method would provide a means for studying the physiological importance of free choline. The organs used in the present study have been those that are considered more important in the metabolism of choline.

The microbiological method of Horowitz and Beadle (2) for the determination of choline has with minor modifications proved very satisfactory in our laboratory. However, before making extensive use of the method we considered it desirable to make a comparison of the values obtained by the microbiological and chemical methods. The results of this study are included in the present paper.

Procedure

Free Choline—A 2 to 3 gm. sample of the fresh tissue is weighed and ground in a mortar. The finely minced tissue is transferred to a 125 ml. Erlenmeyer flask with the aid of 50 ml. of a 1 per cent sodium acetate solution adjusted to pH 4.6. The flasks are then placed in an oven at a temperature of 80° for 1 hour. The solution is then centrifuged and the supernatant decanted. A 5 ml. aliquot of the liquid is transferred to a 15 ml. tapered centrifuge tube containing 10 ml. of acetone. The tubes are then placed in an ice bath for a period of 2 hours and the resulting precipitate removed by centrifugation. The purpose of this acetone treatment is to precipitate any lecithin which may be present. That the precipitate does contain appreciable amounts of lecithin will be shown later in the paper. The liquid portion of the acetone-treated extract is trans-

ferred to a small beaker which is placed over a steam bath until all of the acetone is evaporated. The remaining solution is then made up to a convenient volume, usually 50 ml. 10 ml. of this solution are passed through an adsorption column containing approximately 1 gm. of activated Decalso (60 to 80 mesh). The activated Decalso is prepared in the manner described by Hennessy (3) for thiamine adsorption.

Elution of the choline is effected with 10 ml. of a solution of 5 per cent sodium chloride. The choline-free medium is the same as that described by Horowitz and Beadle (2) with the exception that 1 γ of biotin per liter gives as good growth response as 5 γ which were used in the original medium. The methyl ester was found to be as effective as free biotin when used at a level to furnish equivalent amounts of biotin. *Cholineless* differs from *Lactobacillus arabinosus* in its ability to utilize the methyl ester of biotin, as it is not available to the latter organism (4). Inoculation with *cholineless*, incubation, and measurement of growth response are carried out as described in the original method (2).

Total Choline—For the determination of total choline a 0.2 gm. sample of finely minced tissue is treated with 10 ml. of 3 N hydrochloric acid and autoclaved at 15 pounds pressure for 2 hours. The hydrolysate is neutralized with sodium hydroxide and the whole brought to a convenient volume. An aliquot is now adsorbed on Decalso and the remainder of the procedure carried out as described for free choline.

Horowitz and Beadle (2) used 3 per cent sulfuric acid for the liberation of choline from lecithin. We have compared the results obtained by hydrolyzing with 3 per cent sulfuric acid, saturated barium hydroxide, and 3 N hydrochloric acid. The three hydrolysates on the same tissue gave identical results. Since hydrolysis with hydrochloric acid is less laborious, it has been used routinely.

<center>EXPERIMENTAL</center>

If the total choline content of a tissue could be obtained by adding the free choline plus the choline of the water-extracted tissue after hydrolysis, and the choline obtained by hydrolyzing the precipitate formed by treating the water extract with acetone, it would serve as a measure of the adequacy of the water extraction and acetone treatment. This procedure was followed with four samples of liver. The average results for the four samples expressed in mg. per gm. were as follows: free choline content of water extract 0.12, choline in water-extracted residue after hydrolysis 5.30, choline in acetone precipitate of water extract after hydrolysis 0.42. The total choline content of the liver obtained by adding the three fractions was 5.84 mg. per gm., which differs by less than 3 per cent from the value of 6.07 mg. per gm. obtained by determination of total choline on the fresh liver.

Agreement between the two values is well within the range of experimental error claimed for the method, and affords confirmatory evidence as to the adequacy of the method for free choline.

Experiments were carried out on pure soy bean lecithin to determine whether the lecithin was completely precipitated by the acetone treatment of the water extract. The choline present in lecithin is partially utilized by *cholineless* and so the complete precipitation of it is essential. Known amounts of soy bean lecithin were suspended in water and the procedure followed as previously described for the determination of free choline. The amount of free choline obtained was negligible, showing that the lecithin was completely precipitated by the treatment with acetone.

It seemed desirable to determine whether the values for free choline obtained by extraction with water could be verified by extraction with 70 per cent acetone. Since choline is soluble in 70 per cent acetone, while lecithin is insoluble, the two methods of extraction should give the same values. To test this assumption a 2 gm. sample of minced liver tissue in a fiber extraction thimble were placed in a Soxhlet extractor. The extraction was carried out with 70 per cent acetone for 24 hours. At the end of this period the acetone was evaporated off over a steam bath and the residual solution made up to 50 ml. This solution was then assayed for free choline. The free choline values determined by this method on six samples of liver were not significantly different from the values obtained by extraction with water followed by treatment with acetone, as described in the procedure.

Effect of Adsorption—All non-basic substances which may interfere with the growth of the *Neurospora* are eliminated by the Decalso treatment. Methionine if present in sufficient amounts will be utilized by *cholineless* and thus must also be eliminated (2). The effect of pH on adsorption of solutions containing choline was studied. The results showed that choline is quantitatively adsorbed at a pH of from 4.5 to 7.0. Table I shows the effect of adsorption on the free and total choline values of liver. The values for free choline are very much less after adsorption. Thus it is apparent that there are appreciable amounts of foreign growth substances present in the water extract and that their effect can be eliminated by adsorption. While there is a decrease in the values for total choline with adsorption, it is not nearly so pronounced as in the case of free choline. Nevertheless the difference is sufficient to warrant adsorption in the determination of total choline.

Standard solutions of choline chloride adsorbed on Decalso are eluted quantitatively with a solution of 5 per cent sodium chloride.

Recovery of Choline Added to Tissue—Known amounts of choline chloride were added to minced beef liver and assays made to determine the recovery. Both total and free choline were determined on each sample of liver. The

amount of choline added for the free choline determination would of necessity be much smaller than the amount added for the total choline determination. For this reason four samples were taken from each liver. Two samples were necessary for the total choline; one served as a control and to the second a known amount of choline chloride was added. Two samples were used in a similar manner for free choline determinations. The average recovery in the free choline determination was 98.2 and 98.0 per cent

TABLE I

Effect of Adsorption on Free and Total Choline Values of Fresh Liver

	Free choline		Total choline	
	Adsorbed	Not adsorbed	Adsorbed	Not adsorbed
	mg. per gm.	*mg. per gm.*	*mg. per gm.*	*mg. per gm.*
Average of 10 samples	0.12	1.03	6.50	7.25
Range . ..	0.08–0.20	0.75–1.51	5.83–7.02	6.74–8.28

TABLE II

Recovery of Choline Added to Beef Liver

Sample No.	Type of choline	Choline content of sample	Choline added	Total	Found	
		mg per gm	*mg. per gm*	*mg. per gm.*	*mg. per gm*	*per cent*
1	Free	0.13	0.20	0.33	0.30	91
	Total	6.61	10.00	16.61	16.40	99
2	Free	0.10	0.50	0.60	0.62	103
	Total	6.42	15.00	21.42	20.63	96
3	Free	0.18	0.80	0.98	1.04	106
	Total	6.86	20.00	26.86	26.80	100
4	Free	0.08	0.90	0.98	0.90	92
	Total	5.95	25.00	30.95	30.00	97
5	Free	0.14	1.00	1.14	1.13	99
	Total	6.76	30.00	36.76	36.16	98

for the total choline (Table II). These figures are well within the limits of experimental error ascribed to the method.

Comparison with Chemical Methods—No information has been reported comparing the microbiological and chemical methods for the determination of choline. It seemed desirable to make such a study, and two chemical methods were selected, both of which involved the precipitation of choline as the reineckate according to Beattie (5). The first method was that of Engel (6) and the other of Marenzi and Cardini (7). The microbiological and chemical determinations were made on beef liver which had been dried at 105° for 24 hours. From the data in Table III it is obvious that the three

methods give essentially the same value for, choline when applied to liver. There is no reason to expect that the agreement would not be equally good with other tissues.

Determination of free choline in liver by the chemical methods did not prove satisfactory. This was due apparently to their relatively lower sensitivity as compared with the microbiological method.

Effect of Ingestion of Choline on Tissue Content—Some information was secured on the amounts of total and free choline in tissues as influenced by the ingestion of choline. The tissues studied were liver, kidney, and brain.

TABLE III

Total Choline Values of Dried Beef Liver Determined by Microbiological, and Chemical Methods

Method	No of determinations	Choline content	
		Average	Range
		mg per gm	*mg. per gm*
Microbiological.	4	18.60	17.97–20.21
Engel	4	18.04	17.50–18.87
Marenzi and Cardini	3	19.13	18 13–20.07

TABLE IV

Effect of Diet on Choline Content of Tissues of Rats

Dietary treatment	Rat No.	Liver		Kidney		Brain	
		Free	Total	Free	Total	Free	Total
		mg per gm	*mg per gm*	*mg per gm*	*mg. per gm*	*mg per gm*	*mg. per gm.*
Control ..	1	0.10	9.50	0.13	3.75	0.15*	4.25*
" ...	2	0.07	6.00	0.17	6.00		
Fed choline	3	0.06	7.00	1.60	5.25	0.12*	3.82*
, " " .	4	0.12	6.25	2.37	6.42		

* Composite sample from two rats.

Mature rats were fed a standard diet to which 1 per cent of choline had been added. The duration of the feeding period was 8 days. Table IV gives the free and total choline values of the liver, kidney, and brain of two rats fed the ration containing choline and similar data for rats fed the standard diet without the choline. The only striking difference occurred in the free choline content of the kidney. The free choline content of the kidney of the rats fed choline was about 20-fold greater than the values for the controls. The fact that there was no material increase in the free choline content of the liver is rather surprising, as it is generally believed that this organ is the chief site of the metabolism of choline. The increase of free

choline in the kidney suggests that this organ may play a rôle in the metabolism of choline. However, its interpretation must await further studies.

SUMMARY

A microbiological method is described for the estimation of free choline in animal tissues.   Values for free choline in fresh beef liver range from 0.08 to 0.20 mg. per gm.   This represents about 2 per cent of the total choline in the liver.

For the determination of total choline the values by the microbiological method agreed very well with the values obtained by chemical methods.

Incorporation of 1 per cent of choline in the diet of the rat is accompanied by a rather large increase in the free choline content of the kidney.   Liver and brain tissue showed no corresponding increase.

BIBLIOGRAPHY

1. Luecke, R. W., and Pearson, P. B., *J. Biol. Chem.*, **153**, 259 (1944).
2. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943)
3. Hennessy, D. J., *Ind. and Eng. Chem.*, *Anal. Ed.*, **13**, 216 (1941).
4. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941)
5. Beattie, F. J. R., *Biochem. J.*, **30**, 1554 (1936).
6. Engel, R. W., *J. Biol. Chem.*, **144**, 701 (1942).
7. Marenzi, A D., and Cardini, C. E., *J. Biol. Chem.*, **147**, 363 (1943).

MODIFIED ELECTROLYTIC GUTZEIT METHOD FOR RAPID MICRODETERMINATION OF ARSENIC*

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An investigation requiring the accurate determination of minute amounts of arsenic in cerebrospinal fluid led to the further modification of the electrolytic Gutzeit method previously reported by the senior author (1).   The present method allows the determination of 1 to 100 γ of arsenic with an error of 5 per cent or less.   The per cent of error is increased to between 5 and 10 per cent when the values dealt with are between 0.1 and 1.0 γ.   The apparatus (Fig. 1) and procedures are relatively simple and as many determinations as one has electrolytic chambers for can be made in about an hour and a half.

The method consists of a preliminary sulfuric acid digestion of the sample and reduction with stannous chloride.   Electrolysis of the acid solution is utilized to liberate arsine.   After passage of the arsine through glass wool impregnated with lead acetate, it stains a mercuric chloride-sensitized string to a length accurately proportionate to the amount of arsenic present.

The mercury bromide-sensitized paper strips previously used have been discarded in favor of the mercuric chloride-sensitized string snugly suspended in a capillary tube as described by How (2).   A complete review of the literature is included in his article.   How made a careful and thorough study of the various types of sensitizers, materials used for indicators, and the conditions which gave the most satisfactory results.   With a few variations, the string is prepared and used as recommended by How.   It not only has the advantage of a uniform and sharply demarcated stain but also allows a very broad range of determinations without loss of accuracy.   This is made possible through the use of strings of varying degrees of sensitivity. For such minute quantities as 0.1 to 2.0 γ, strings saturated in 0.25 per cent solution of mercuric chloride are used; for 1 to 10 γ, strings saturated in 1

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per cent solution of mercuric chloride are used; and for 5 to 100 γ, strings saturated in 5 per cent solution of mercuric chloride give the most satisfactory length of stain.

Reagents completely free of arsenic are desirable and, even if these are available, blanks should be run at frequent intervals, since the minute traces from the glassware, apparatus, and the so called arsenic-free reagents are detectable with the more sensitive strings. When samples containing relatively large amounts of arsenic, for example 50 to 100 γ, are run, detectable traces remain in the chamber despite thorough washing. These can be removed only by continuing electrolysis with blank reagents in the chamber for a number of hours.

Following digestion, How reduced the pentavalent arsenic to the trivalent state by the addition of 0.1 gm. of sodium bisulfite to the sample, heating for 30 minutes on an 80–85° water bath, and then removing the remaining sulfur dioxide by boiling for 3 minutes. The sodium bisulfite is unstable in aqueous solution and therefore 0.1 gm. amounts of the powder must be weighed individually. An additional objection is the relatively rapid exhaustion of the lead acetate scrubber by the traces of remaining hydrogen sulfide which is produced. Equally satisfactory reduction can be obtained by adding 3 drops of a 60 per cent solution of stannous chloride in concentrated hydrochloric acid and boiling for only 3 minutes.

How's method requires meticulous and time-consuming preparation of the zinc alloy generator which, however, is reported to give consistently uniform results. The advantage of the electrolytic chamber lies in its relative simplicity of construction and use. The apparatus can be made by the average glass-blower. It operates on a direct current of 1.0 ampere and 12.0 volts, and any desired number of the electrolytic chambers can be connected in series.

Instead of the V-shaped cathode and anode chambers separated by an alundum disk as previously described, the apparatus now used has a sealed-in porous glass disk at the base of the cathode chamber which is conveniently suspended within the anode chamber. When the apparatus is set up so that the fluid level in the anode chamber is slightly higher than that in the cathode chamber, diffusion of arsenous acid out of the latter is prevented and complete and rapid reduction of the arsenous acid to arsine occurs. The gases evolved from the electrolytic chambers have a high water vapor content but a more uniformly high saturation is insured through the insertion of a water saturation tube as shown in Fig. 1. An actual water trap should not be used with the electrolytic apparatus, since it is not a completely closed system and a trap alters diffusion through the porous glass disk.

The electrolytic chamber rests in a water bath which during operation is

maintained at a temperature of about 5° above the temperature of the capillary absorption tube, which is at room temperature. As emphasized by How, this temperature ratio gives the sharpest stain end-point, a fact probably attributable to water vapor condensation.
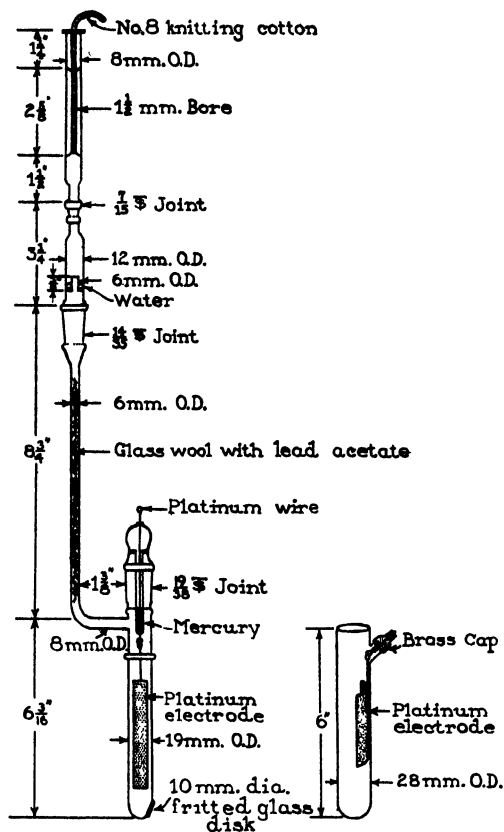
FIG. 1. Diagram of electrolytic Gutzeit apparatus

Apparatus and Reagents

The apparatus consists of Pyrex glass and petrolatum-lubricated ground glass joints of the dimensions noted on the diagram. The cathode consists of a lead tube folded from a sheet, or preferably of a platinum tube, since all lead sheet at our disposal contains traces of arsenic. The anode is a small sheet of platinum bent to conform to the curvature of the chamber. The cathode is fastened to the glass stopper by a sealed-in platinum wire connected through mercury to a copper wire contacting the source of current. Short pieces of insulated copper wire with terminal clamps serve as connec-

tions. Amperage of the direct current is controlled by a rheostat connected in the circuit.

The electrolytic chambers are placed in a metal rack resting in a water bath with inlet at the bottom and drainage near the top. A thermometer is suspended in the water bath. Adequate temperature control is effected through regulation of the rate of water flow.

The lead acetate-impregnated glass wool for removal of hydrogen sulfide gases is prepared by soaking the glass wool for several hours in 10 per cent solution of lead acetate. The excess solution is expressed and the glass wool placed on a towel to dry in the air. Strands of the glass wool are then loosely packed into the long side arm stem.

The water vapor chamber should contain 0.2 cc. of distilled water and can be filled from the top with a pipette.

The string (No. 8 knitting cotton) can be prepared by winding 20 to 50 feet of it in closely spaced spirals around a glass cylinder or tube. A 500 cc. cylinder is satisfactory. The alcoholic solution of mercuric chloride of the desired concentration (0.25, 1.0, or 5.0 per cent) is prepared with 95 per cent alcohol. The wound tube is immersed in the solution which has been poured into a cylinder large enough to accommodate the wound tube. Although How recommended soaking for many hours, we have found 1 hour of soaking to be adequate, although longer soaking does no harm. Upon removal of the wound tube from the solution, the entire string is drawn off, held slightly taut, and rotated in a horizontal position while it dries for 5 minutes. With a minimal amount of handling the string is cut into 10 to 12 cm. pieces, which are stored in a covered glass tube or chamber protected from the light with black paint or paper. Despite apparent uniformity of technique in preparation of strings of the same concentration, slight differences in sensitivity occur between the different lots, so that for greatest accuracy each lot must be standardized individually.

A 2 per cent solution of silver nitrate in a 10 per cent solution of ammonium hydroxide is used to develop the black stain on the exposed end of the string.

The 60 per cent solution of stannous chloride solution is prepared by dissolving 30 gm. of the crystals in concentrated hydrochloric acid made up to 50 cc.

12.5 per cent sulfuric acid is used to half fill the anode chamber for electrolysis.

Concentrated sulfuric acid and a 10 per cent solution of copper sulfate are used in the digestion process.

Procedure

For the analysis of a biological fluid such as spinal fluid, add 5 cc. of concentrated sulfuric acid and 0.5 cc. of 10 per cent copper sulfate solution to

2 cc. of spinal fluid in a Kjeldahl flask. Boil in a hood or on a Kjeldahl digestion rack for 30 minutes and then cool. If blood, feces, skin, or other tissues are being analyzed, use 10 to 20 cc. of sulfuric acid and 1 cc. of copper sulfate solution and continue the digestion for an hour.

Dilute the digested mixture to 50 to 100 cc. and use an aliquot part for electrolysis, the quantity of the aliquot depending on the amount of arsenic expected to be present. A 20 or 25 cc. aliquot part is usually satisfactory.

Add 3 drops of the 60 per cent stannous chloride solution and two Pyrex glass beads to the aliquot part in a 100 cc. Erlenmeyer flask and boil for 3 minutes. Cool.

Fill the anode chamber with 12.5 per cent sulfuric acid to about the half full mark. Place the cathode chamber down in the anode chamber and after 1 to 2 cc. of acid have diffused up through the porous glass disk pour the contents of the flask into the cathode chamber. Rinse the flask with 1 to 2 cc. of distilled water from a wash bottle and add the washing to the cathode chamber. Ideally at this point the fluid level in the outer anode chamber should be about 1 cm. higher than in the inner cathode chamber. The cathode and its glass stopper are lowered into place, sealing the chamber which is placed in the water bath.

The capillary absorption tube containing the sensitized string, previously placed in position by applying slight suction, is now affixed to the water vapor chamber which in turn can be fitted into its joint at the top of the long side arm. The direct current terminals are clamped to the electrodes and the electrolysis continued for 30 minutes.

After this time the sensitized string is withdrawn from its tube and the lower end is dipped into the 2 per cent solution of ammoniacal silver nitrate. A deep, black, sharply demarcated stain instantly develops over the end of the string. The length of stain is measured with calipers or with a millimeter rule against a white background.

Preliminary tests with known amounts of arsenic give data for tables or curves, so that the calculation of the amount of arsenic in the sample depends merely on the amount of original material digested, the amount of dilution, and the size of the aliquot part. For example, 2 cc. of spinal fluid diluted after digestion to 50 cc., of which 25 cc. have been used as the aliquot part, give a stain 5.0 mm. long on a string saturated in 0.25 per cent solution of mercuric chloride. Tabulated data from preliminary "known" analyses show that a 5.0 mm. stain on the 0.25 per cent string of that lot is equivalent to 1.0 γ of arsenic. The sample of spinal fluid therefore contained 1.0 γ of arsenic per cc.

Representative samples of sensitized string saturated with a 1 per cent solution of mercuric chloride have in our hands given the following lengths of coloration with the various quantities of arsenic: 1 γ, 1.0 mm.; 3 γ, 3.0 mm.; 7 γ, 6.8 mm.; 10 γ, 9.8 mm.

SUMMARY

We have described an apparatus and technique which combine the use of an electrolytic Gutzeit device with certain features of the How method for the determination of minute amounts of arsenic in biological material. The accuracy of the method is of the order of 10 per cent for amounts of arsenic from 0.1 to 1.0 γ and is increased to 5 per cent with amounts of arsenic from 1.0 to 100 γ.

BIBLIOGRAPHY

1. Osterberg, A E , *J. Biol Chem.*, **76,** 19 (1928).
2. How, A E , *Ind. and Eng Chem , Anal. Ed.*, **10,** 226 (1938).

IDENTIFICATION OF SMALL AMOUNTS OF ORGANIC COMPOUNDS BY DISTRIBUTION STUDIES

II. SEPARATION BY COUNTER-CURRENT DISTRIBUTION

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In a previous publication (1), a method for the identification of extremely small quantities of organic compounds through the use of distribution data has been proposed. The procedure given should be quite reliable and sensitive for certain types of compounds, such as the antimalarial drug atabrine, for the reason that in this particular case the curves derived are functions not only of the distribution constants in a series of solvents, but also of the dissociation constants and the fluorescent capabilities as well. Atabrine or any other compound of acid or basic character which can be studied spectroscopically is thus particularly well suited for such a study.

However, many of the compounds of pharmacological and biological interest or their metabolic or detoxification products do not have pronounced basic or acidic groups, and are not suited for convenient spectroscopic study with the methods at present available. The absence of either one or both such favorable properties obviously lowers the reliability of conclusions which might be drawn from distribution studies, unless some method is employed which is capable of greatly enhancing the significance of the distribution data. A search has been made for some way of accomplishing the latter, and also for some method of making use of distribution data to bring about actual separation of submicro amounts when the data indicate mixtures to be present.

In more recent years, a very useful and important technique has been developed for the separation of complex mixtures by the use of the relative distribution of the constituents between two liquid phases, and has come to be known as "counter-current extraction" (2–4). If this type of procedure could be modified in a manner to make it more practical for the study of the extremely small amounts with which this investigation is concerned, it would appear to offer the most promising approach to the problem stated above. The literature of interest, from our particular view-point, is well summarized in a paper by Martin and Synge (5).

Also of especial interest in this connection is the outstanding contribution of Martin and Synge (6), in which a new form of chromatogram for which two liquid phases are employed has been developed. One, an aqueous phase, was immobilized in silica gel. Unquestionably, their beautiful

technique could offer precisely the tool needed for many aspects of our problem. However, it would appear to have certain limitations in further exploiting the approach suggested by the previous publication (1), particularly when mixtures of different solvents and buffered solutions are employed.

Irrespective of the possibilities inherent in the method of Martin and Synge, an entirely different type of apparatus, rather simple in design, has been developed in this laboratory. It would appear to lack certain features of the large scale equipment for continuous fractionation, but has certain admirable features from an analytical standpoint.

It was constructed, as shown in Fig. 1, by Mr. Otto Post of our laboratory. We are greatly indebted to him for the especially precise work, for the many valuable suggestions, and for technical assistance throughout its development.

The main part of the apparatus was made from the largest stock of stainless steel which could be obtained at the time, approximately 11.4 cm. in diameter. This was cut into three parts, 8, 10.5, and 1.3 cm. long. The one 8 cm. long formed the lower part, A, of the apparatus. Twenty holes, each 12 mm. in diameter and 6 cm. in depth, were drilled in A so that they formed an exactly symmetrical arrangement of tubes in a circle around the outer portion of the cylinder. Each hole was 3.6 mm. from the next and was 3.6 mm. from the outer edge of the cylinder. Each one would contain exactly the same volume. Most of the metal in the center 6 mm. from the inside edges of the holes was removed, in order to make the apparatus lighter. A layer approximately 3 cm. thick was left for the support of the central stem, E, which holds the apparatus together.

The part 10.5 cm. in length formed the central part, B, of the apparatus. Holes of the same diameter and spaced exactly the same as in A were similarly drilled, but in this part extended entirely through the section. Here again, most of the central part of the metal was removed, with the exception of a layer approximately 2 cm. thick which formed a bearing through which the central stem, E, could pass.

The part 1.3 cm. in length formed the cover, C. A layer of the metal approximately 2 mm. in depth was removed from the central portion of the under side at a distance of 22 mm. from the outer edge of the plate. A hole just large enough to allow the passage of E was then drilled in the center. Another hole 10 mm. in diameter and threaded to receive the plug, H, was drilled so that its outer edge was 4 mm. from the outer edge of the plate. The liquid in any one of the tubes could be withdrawn at will by removing H.

The central stem, E, was made from a stainless steel rod 13 mm. in diameter. Its lower end was attached to A exactly in the center by a thread,

so that it stood parallel with the long axis of the apparatus. The stem extended through a hole drilled in the center of B and through a similar hole in C. It extended approximately 4 cm. above the cover, C, and had a thread at the top. A large wing-nut, F, was used to hold the three parts firmly together. A rather strong coil spring, G, was placed just under the wing-nut, F.
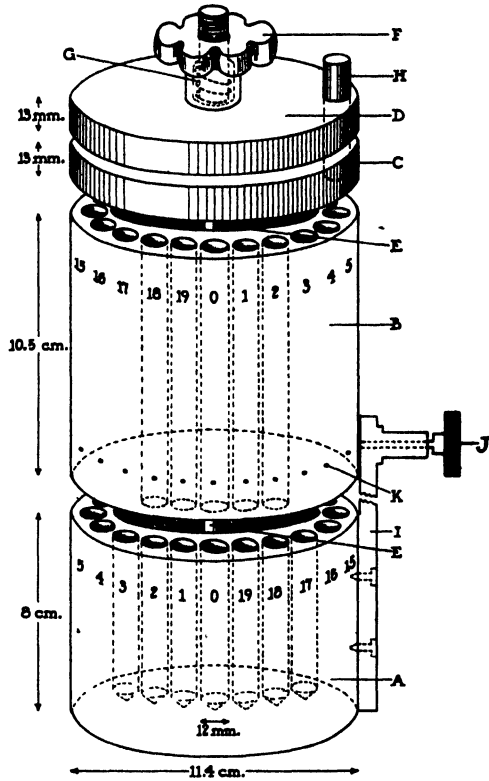
FIG. 1. Counter-current distribution apparatus

The surfaces between A and B, and between B and C were accurately ground to prevent leakage in a manner similar to the way desiccator tops are fitted. However, the cover, C, was found to bend just enough to cause leakage when pressure was applied to it from the wing-nut, F, and it was found necessary to place over C another similar cover, D, made in the same way, but not necessarily accurately ground, so that the pressure would be applied directly to the outer edges of the cover, C. When properly cleaned and used under constant temperature conditions, very little leakage occurred at the ground joints.

A piece of metal, I, was securely fastened by means of screws to A at the position shown. The upper part of this carried an indicator plug, J, such as that used on milling machines. J had a small coil spring attached, which forced it into the small holes, K, accurately placed on the outer surface of B in such a position that the tubes in A and B were exactly over each other when the plug entered the small hole. The part B could be thus rotated around the stem, E, and stopped at any desired position with the tubes of A and B exactly coinciding. The upper tubes were numbered counter-clockwise, starting with 0, and the tubes in A were numbered clockwise, starting with 0.

The apparatus, when put together, thus contains a total of twenty tubes 12 mm. in diameter and 16.5 cm. in length which, for purposes of theoretical discussion, may be considered as a series of separatory funnels. For operation, two liquids mutually saturated with each other are placed in each tube with the volume of the heavier layer always fixed at just sufficient to fill the A part of the tube. The meniscus dividing the two layers is thus always at the point where it can be cut by rotation of the part B, with A remaining stationary. The operation of filling the lower half of the tubes can be quickly accomplished by adjusting the part B so that the tubes are midway from coincidence with the tubes of A. Each tube of B will then connect with two tubes of A, and all the tubes in the apparatus will be connected with each other. The lower tubes can be filled by pouring 130 cc. (6.5 cc. for each tube) of the heavier liquid into one of the tubes at the top. A few turns of B on A then equally distribute the fluid so that it just fills all the tubes of the part A.

After upper Tube 0 is brought over lower Tube 0, 6.5 cc. of the lighter layer are added to each of the tubes. There will now be an air space 4.5 mm. long above the surface of the liquid, which is sufficient to allow for shaking after the top is sealed with the cover, C. The sample of the substance to be distributed is placed in Tube 0. Shaking is accomplished by quickly inverting the apparatus again and again at a speed which keeps the liquid constantly in motion. The whole assembly is somewhat too heavy for a shaker and, because of the rather narrow tubes, inversion with the use of a bearing for support proved the best way of shaking. In order to control the speed of inversion, a glass tube of exactly the same inside dimensions as the ones in the apparatus is filled with the same volumes of the two layers and attached to the outside of the apparatus by means of rubber bands. The optimal manner of shaking and handling of the apparatus can best be governed by observing the behavior of the two fluids in this tube.

After being shaken (from 0.5 to 2.0 minutes, depending on the ease of emulsification, has proved sufficient to reach essential equilibrium), the apparatus is placed upright and allowed to rest until the two layers separate. The wing-nut, F, is then released slightly so that the parts are held together

only by the force of the coil spring, G, and the indicator plug, J, is pulled out. The upper numbers are advanced slightly by rotating B clockwise on A, and the indicator plug is allowed to spring in again. When B is rotated further on A, the indicator plug will snap into position, and at this point upper Tube 0 will be exactly over lower Tube 1. The shaking, etc., can then be repeated until upper Tube 0 is over lower Tube 19. At this stage, nineteen equilibriums or "plates" have been accomplished. In this paper, the term plate will be applied to each one of the stages as described.

The term plate, as used in this paper, is not the same as the concept of "theoretical plate," as used by Varteressian and Fenske (3) and others in counter-current extraction work. The degree with which the former approaches a theoretical plate would appear to depend on a number of factors, such as whether or not equal volumes of liquid are present in the upper and lower layers, the extent to which complete equilibrium is reached, the sharpness of separation of the two layers, etc.

After the point in the procedure has been reached at which upper Tube 0 is over lower Tube 19, a number of different ways of proceeding may be followed, depending on the purpose to be accomplished and the type of mixture. Some of these might be as follows:

1. All the fluid, both upper and lower layers, of which lower Tube 19 is a part, may be siphoned out through the opening in C, and investigated by some means to learn whether any of the substance being studied has reached this tube. If not, the solvents can be replaced and more plates applied until an appreciable amount of the material has reached the tube mentioned. The total number of plates applied is then recorded, and each of the twenty tubes investigated by some means, such as titration, spectroscopic analysis, evaporation to dryness, etc., to learn what percentage of the total employed in the beginning is present in each tube.

2. Procedure 1 may be continued by withdrawing the material in the tube of which lower Tube 19 is a part, setting it aside, replacing the fluid with exactly the same volumes of the two layers with fresh solvent, and progressing one more plate. This procedure can be repeated indefinitely until most of the material with the higher distribution constant has passed from the apparatus. Finally, those first obtained, together with the twenty remaining in the machine, are investigated to determine the amount in each tube.

3. Procedure 1 may be followed until nineteen plates (more or less, if desired) have been applied and the distribution has been learned by investigation of the amount in each tube. Certain strategic fractions of optimal percentage can then be combined, the solvent removed, and the whole operation repeated on the selected mixture. The procedure can be repeated indefinitely until the desired separation has been achieved.

Other procedures which are variations of these naturally may be followed.

It appears scarcely advisable to consider them here, but rather to investigate somewhat the possibilities and the limitations of the three given. Furthermore, it seemed wise in the beginning to establish definitely the degree to which a pure substance is distributed, or travels around the circle of tubes, according to mathematical calculation. For this latter purpose, β-naphthoic acid was chosen.

On general grounds, it appeared desirable to choose two liquids for the immiscible solvents which would give a distribution constant not far removed from 1, in order to gain the most satisfactory view of the performance of the apparatus. A wide variation in the distribution constant can obviously be achieved through the use of mixed solvents. It was found that a mixture of 50 per cent benzene-50 per cent hexane for the upper layer, and 80 per cent methanol-20 per cent water for the lower layer (mutually saturated with each other) gave a distribution constant at room temperature of approximately 0.391, a figure within the desired range. Procedure 1 was, therefore, applied to 188 mg. of the acid in the solvents mentioned. Since a known, pure substance was taken at the beginning and some hint of its rate of travel could be implied from the distribution constant, no investigation of the amount in the tube over lower Tube 19 was made after nineteen plates had been applied. Instead, more plates were applied until a total of 59 had been reached. Upper Tube 0 had thus passed around the circle of lower tubes two times and further, until it was again over lower Tube 19. The material in each tube was then removed and titrated with standard 0.1005 N sodium hydroxide against phenolphthalein.

When the volume of standard alkali consumed was plotted against the number on the lower part of the tube, the strikingly symmetrical curve shown in Fig. 2, Curve 1, was obtained. Here the choice of tube numbers for the abscissa is arbitrary, as long as the tubes are arranged consecutively. It could be considered just as well that the upper part of the tube is stationary and that only the lower part moves counter-clockwise, or it could be considered that each moves an equal distance in opposite directions. The arbitrary convention, in which the lower part of the tube is considered stationary, seems a little more easy to discuss and will be followed throughout this paper.

The general similarity in shape of the experimental curve obtained to that of a Gaussian distribution curve suggested at once that an equation should be possible which is based on the number of plates, the distribution constant, K, and the concentration. This possibility was discussed with Dr. Alexandre Rothen, who pointed out the similarity of the effects obtained to those of linear diffusion against a concentration gradient. We are greatly indebted to him for his suggestions which, in the main, led to the

development given below. Analogy of certain aspects of counter-current extraction to diffusion had been suggested previously by Cornish, Archibald, Murphy, and Evans (2).

Linear diffusion against a concentration gradient is described by the classical Equation 1, developed many years ago by Wiener (7).

$$\frac{dc}{dx} = \frac{C}{\sqrt{4\pi Dt}} e^{-x^2/4Dt} \tag{1}$$

A basis for the theoretical behavior of counter-current distribution in the practical range may be established from a few approximate tables based on the percentage of substance in the upper layer $(K/(1 + K))$, such
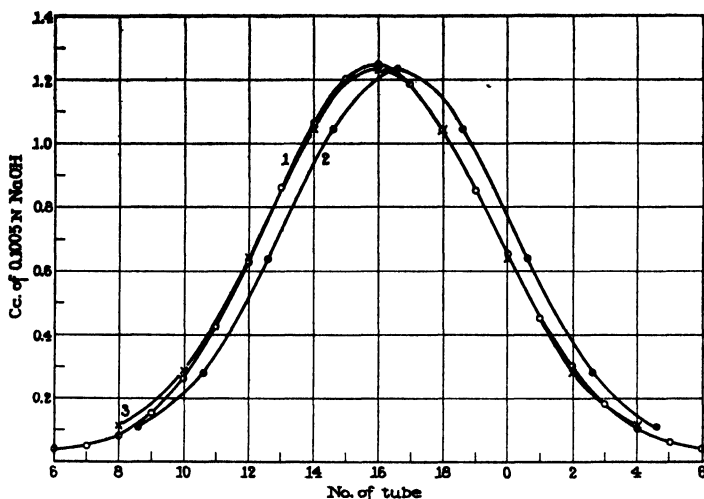
FIG. 2. Curve 1 represents the experimentally determined curve for pure β-naphthoic acid (59 plates); Curve 2, calculated curve; Curve 3, calculated curve with maximum superimposed over experimentally determined maximum

as Table I (binomial expansion (6)). Table I gives the distributions obtained with equal volumes of the light and heavy layers when 1 gm. of substance having a distribution constant of 1 is used. For convenience, the figures of Table I were rounded off to the nearest 0.001 gm. Either by plotting curves at the various plates, or by examination of Table I, it can be derived that the maximum of the distribution curve will migrate from the starting point 0, according to the percentage in the upper layer, Equation 2.

$$N = n\left(\frac{K}{K + 1}\right) \tag{2}$$

In Equation 2, K is the distribution constant, n is the number of plates applied, and N is equal to the number of tubes the maximal concentration has migrated from Tube 0. $K/(K + 1)$ would thus seem to relate the rate of migration of the band to the distribution constant, and might be substituted for the diffusion constant D in Equation 1, when the number of plates applied is also substituted for time. When this was tested out on the theoretical cases from Table I, the relationship was found to hold if $K/(K + 1)$ were multiplied by the constant 0.26.

Other theoretical tables, such as Table I, were calculated for cases in which the distribution constant is other than 1 ($K = 0.5, 0.25,$ and 0.1), and a similar approach was made as in Table I. In this way, it was found that the constant 0.26 did not hold for any of these, but that each had a

TABLE I

Calculated Distribution

The values are given in gm. to the nearest 0.001 gm.

No of plates applied	No on lower half of tube										
	0	1	2	3	4	5	6	7	8	9	10
0	1.000										
1	0.500	0.500									
2	0.250	0 500	0.250								
3	0.125	0.375	0.375	0.125							
4	0.062	0.250	0.375	0.250	0 062						
5	0.031	0.156	0.313	0 313	0.156	0.031					
6	0.015	0 093	0.234	0 313	0.234	0 093	0.015				
7	0.008	0.054	0.164	0.274	0 274	0 164	0 054	0.008			
8	0.004	0 031	0 109	0.219	0.274	0 219	0 109	0.031	0.004		
9	0 002	0.017	0 070	0 164	0.246	0.246	0 164	0 070	0.017	0.002	
10	0.001	0 011	0 044	0.117	0 205	0 246	0.205	0 117	0.044	0 011	0.001

characteristic constant of its own. If, for simplification, this constant is combined with the constant 4 of Equation 1, Equation 3 would result.

$$\frac{dc}{dx} = \frac{C}{\sqrt{a\pi \left(\frac{K}{K+1}\right) n}} \, e^{-x^2/a \left(\frac{K}{K+1}\right) n} \tag{3}$$

Equation 3, therefore, approximates closely the distribution of the material as it migrates around the circle of tubes at the rate expressed by Equation 2, *for the practical range of use.* In Equation 3, C = the amount of substance, K = the distribution constant, a = a constant characteristic of the distribution constant K, n = the number of plates applied (the number of operations), and x = the number of tubes removed from the tube of maximal

concentration. On theoretical grounds, it is to be expected that, for the migration of the band after a true Gaussian distribution had been reached, the constant a would approach 4, and thus the whole phenomenon would become exactly the same as that of diffusion.

Curves calculated from Equation 3 could naturally not be expected to fit experimental curves until enough plates had been applied so that a symmetrical distribution had essentially been reached. The number of plates required for practical application varies with the distribution constant. For an approximation, the minimal number of plates required is given by Equation 4.

$$1 - \left(\frac{K}{K+1}\right)^n = 0.001 \tag{4}$$

For fewer plates, the curves can be calculated from tables or from the binomial expansion calculation.
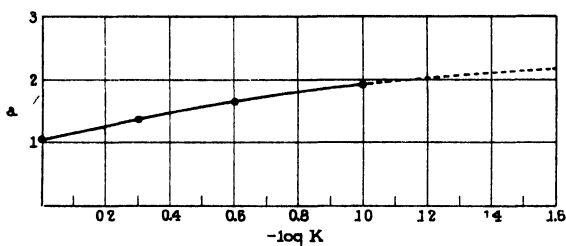
FIG. 3 Relation of constant a to distribution constant K

The constant a can easily be derived from tables such as Table I, but to prepare such tables for each distribution constant encountered would be a very laborious procedure. This objection was easily overcome when it was found from the few tables already prepared that the constant plotted against the negative logarithm of the distribution constant gave the curve as shown in Fig. 3. The constant a is thus easily obtained for any K in a practical range which might be encountered.

Equation 3 cannot be used directly as such when the distribution constant is greater than 1. For these cases, it may be considered that the two phases are inverted, or that the lower part, A, of the apparatus is the one which is rotated. Thus it is permissible to use $1/K$ instead of K for the calculation of the distribution curve, when K is greater than 1.

Most of the development given above could have been derived-from the excellent theoretical treatment of Martin and Synge (6) for the migration of substances in their chromatogram. However, many less factors are involved in the practical use of our apparatus, and a more direct development with fewer assumptions is possible. This favorable aspect of the apparatus

also allows the data to be applied quantitatively to mixtures in which the different bands migrate independently of each other.

In order to check Equation 3 experimentally, or conversely in order to check the efficiency and manner of handling the apparatus, a curve was calculated for a pure substance with a distribution constant of 0.391 when 59 plates were applied to it. The curve shown in Fig. 2, Curve 2, was so obtained. As can be seen from the curves when their maxima are super-imposed, the agreement in shape and thus in distribution with the experimentally determined curve is quite striking. However, the maximum of the experimentally determined curve as actually found is displaced toward the left. The relative amount of this displacement would appear to offer a suggestion as to the over-all amount of divergence from perfect performance due to such factors as change of temperature, slight emulsions, mechanical imperfections, improper equilibration, etc. It scarcely need be said that the ability to calculate a distribution curve will prove of very great importance in interpretation of experimental data and in deciding the optimal conditions under which to effect separation of mixtures.

With the foregoing information at hand, the problem of the separation of a mixture of two substances becomes an interesting one. If their two distribution constants are known, the exact type of curve to be expected can be calculated. It was found experimentally that substances with the ratio of their distribution constants greater than 4 could be separated very easily by Procedure 1 or 2, and it seemed wise to study the case in which the constants are very close together. For this purpose, the pair of substances, α- and β-naphthoic acids, proved especially suitable. In the mixed solvents used above in the study of β-naphthoic acid, α-naphthoic acid proved to have a constant of approximately 0.362. The ratio is, therefore, 1.08.

Calculation of the two independent curves for a 59 plate separation (equal amounts of each acid) gave Curves 1 and 2, Fig. 4. If it is assumed that the two substances migrate independently of each other, the values obtained should be directly additive to give the theoretical curve of the mixture, Curve 3. Curve 4 was obtained experimentally. Here again the shape of the curve obtained experimentally is very similar to that of the theoretical, but its maximum is displaced because of inefficiencies. The two bands would appear to migrate independently of each other, unless a possible association product has a distribution constant very close to the acids themselves. The degree of separation obtainable in each of the fractions can be estimated from the theoretical curves. Fractions 2, 3, and 4 should contain roughly 63 per cent of the β isomer.

Mixtures of two substances were also studied in which the ratio of their distribution constants was intermediate between 4, a mixture very easily separated, and 1, a mixture impossible to separate. p-Toluic and benzoic

acids have the constants 1.65 and 0.68, respectively, a ratio of 2.48, at room temperature in the two phases, 50 per cent benzene-50 per cent hex-
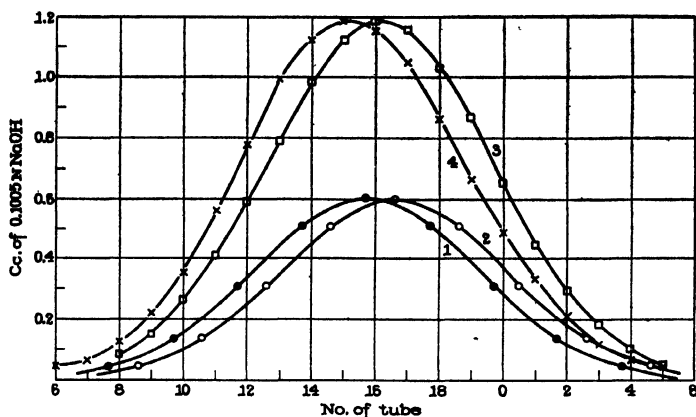
FIG. 4. Curve 1 represents the calculated curve for α-naphthoic acid; Curve 2, calculated curve for β-naphthoic acid; Curve 3, sum of the calculated curves; Curve 4, experimentally determined curve for equal parts of the two acids.
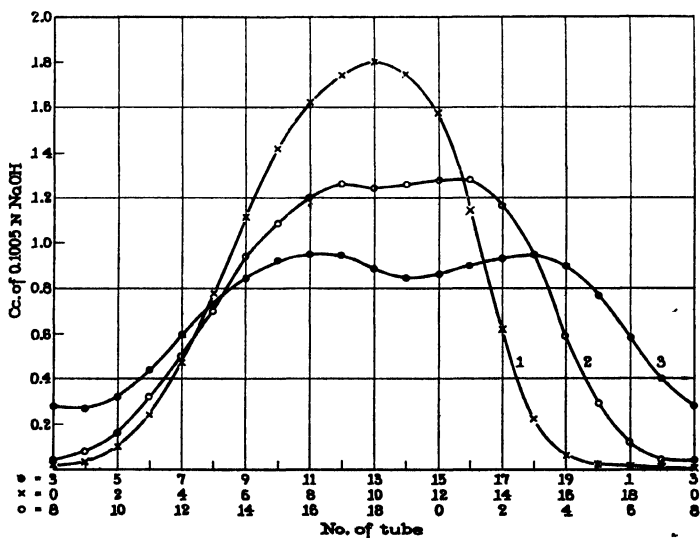
FIG. 5. Distribution of a mixture of equal parts of benzoic and p-toluic acids. Curve 1, nineteen plate separation; Curve 2, twenty-eight plate separation; Curve 3, forty-two plate separation.

ane, and 50 per cent methanol-50 per cent water. Fig. 5 shows the manner in which the two bands emerge as increasing numbers of plates are applied.

Since there are only twenty tubes in the apparatus for the material to be distributed in, overlapping is apparent in Fig. 5, Curve 3, for the larger number of plates, owing to the more rapidly advancing band overtaking the more slowly moving one before separation is accomplished. When Procedure 2, given above, was followed in order to prevent this happening, and fractions were withdrawn after nineteen plates had been reached, the separation given in Fig. 6 was obtained. Here the numbering of the plates is different than in the convention employed with Procedure 1, and the first fraction withdrawn is considered Tube 1, the second Tube 2, etc. The operation was interrupted when 53 plates had been applied. The solution in the tube of which lower Tube 19 is a part was then considered Tube 35, the next to it in a counter-clockwise direction was considered Tube 36, etc. The curve is naturally discontinuous because each of the latter nineteen fractions (Nos. 36 to 55) has had the same number of plates applied, while the earlier fractions are derived only from the upper layer and have had an increasing number of from nineteen to 53 plates applied.

The quantitative aspects of this type of separation are much more complicated to derive mathematically and are scarcely warranted here. That a considerable separation has been achieved is at once apparent from the melting points given with the curve. This type of procedure may prove useful for separating mixtures of substances whose distribution constants are relatively far apart.

If Procedure 1 is followed and twenty-eight plates are applied, as given in Curve 2, Fig. 5, the curve for a theoretical performance can be calculated, as was done for the mixture given in Fig. 4. However, there was found not to be such close agreement between the calculated curve and the experimentally determined curve of the mixture. In the latter, the toluic acid band showed a somewhat lower rate of migration, and there was an obvious distortion. When, however, the two distribution constants were derived from the two maxima of the experimentally derived curve, they were found to be 1.44 and 0.60, respectively. The theoretical curves calculated for these constants are given in Fig. 7. The sum of the two gave Curve 3. Close agreement was not obtained with the experimentally determined Curve 4, and from this it may be suspected that there is a certain degree of association in one of the phases between the two acids, or some such interfering phenomenon, and that the two bands do not migrate with entire independence of each other. A further suspicion that this might be the case was derived from the behavior of the two acids when distributed between the two phases, hexane and 50 per cent methanol-50 per cent water. In the latter case, the undesired effect proved to be much more pronounced, and now the toluic acid band appeared to migrate as scarcely more than half its calculated rate, as contrasted with the behavior of the benzoic acid band, which occurred approximately in the correct position. This is a

point which will be taken up in a later paper when more experience has been obtained. Such phenomena were not unexpected.

In the case of the separation represented in Curve 4, it is probable that the variation from the theoretical is not great enough to interfere seriously with a practical separation, and that the relative amounts in each fraction can be approximately derived from Curves 1 and 2, and will not prove greatly divergent from that actually present in most of the fractions. This is supported by the melting points given in Fig. 6.

In mixtures of substances in which the ratio of their distribution constants is less than 4, Procedure 3 is of greatest interest. As the ratio becomes smaller, more and more plates must be applied to cause the bands to separate. This, however, also causes the bands to broaden and become more diffuse, according to Equation 3. Return of the material of most favorable composition to Tube 0 helps to offset this.
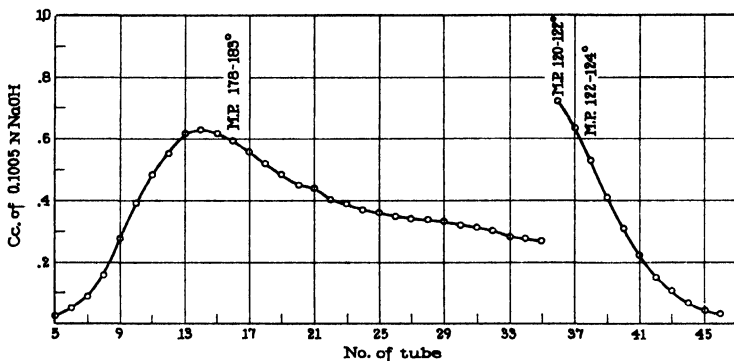
FIG. 6. 53 plate separation of benzoic and p-toluic acids, according to Procedure 2

The maximum separation obtainable can best be pointed out by the use of the following theoretical problem. If a mixture of 1 gm. of Substance A, with a distribution constant of 1, and 1 gm. of Substance B, which has a distribution constant of 0.5 (ratio = 2), is subjected to a nineteen plate separation, the degree to which separation has been achieved can be derived from the calculated curves of Fig. 8. If, at this point, the first seven tubes are combined and the solvent is evaporated, a mixture will result which will contain 80 per cent of Substance B. 0.717 gm. of Substance B, or 71.7 per cent, will have been recovered.

If this mixture is then subjected to another nineteen plate separation, and the first seven tubes are taken, the same relative enrichment will result, and a mixture can be obtained which now has 94 per cent of Substance B in it. However, it will contain only 0.515 gm. of Substance B, or 51.5 per cent recovery.

If the resulting second mixture is subjected to a nineteen plate separation

and the first seven tubes are evaporated, a preparation will be obtained
which will be 98.4 per cent pure with respect to Substance B, but will con-
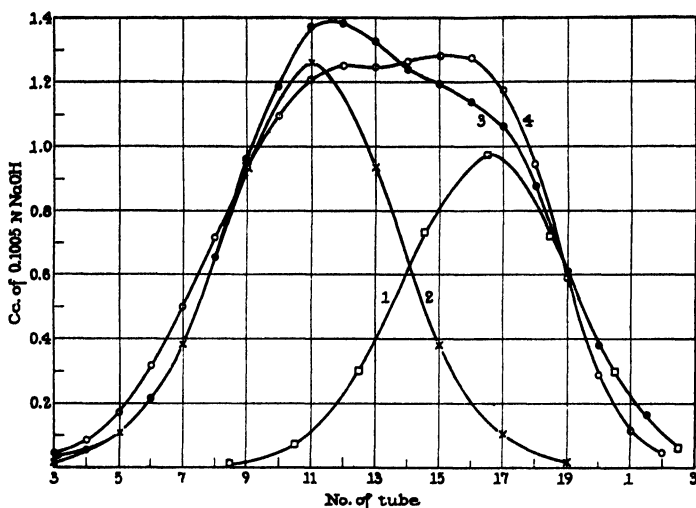
Fig. 7 Curve 1 represents the calculated curve for p-toluic acid ($K = 1.44$); Curve
2, calculated curve for benzoic acid ($K = 6\ 0$), Curve 3, calculated curve for a mixture
(sum of Curves $1 + 2$), Curve 4, experimentally determined curve of the mixture.
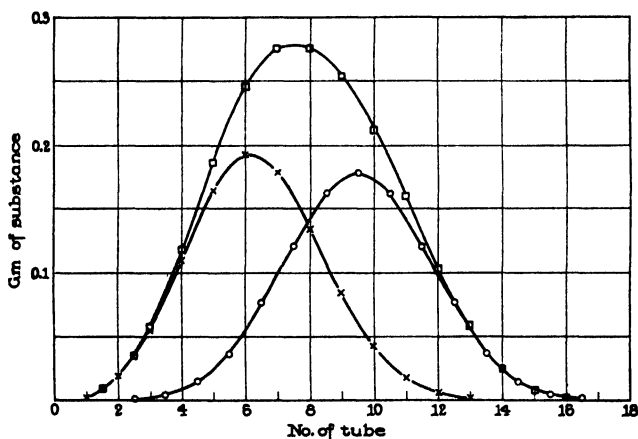
Fig 8 ⊙ represents the calculated curve ($K = 1.0$); ✕, calculated curve ($K = 0.5$); ☐, curve of the mixture.

tain only 0.370 gm.   The over-all procedure, therefore, gives 37 per cent
recovery of Substance B at a purity of 98.4 per cent.   The small divergence
in the rate of migration of the bands thus far studied in the apparatus,

from the theoretical, would indicate that the actual separation obtainable for an ideal mixture, such as given above, would not be far from that given.

Finally, since this apparatus was developed with a definite purpose in mind for biological work, the foregoing treatment would scarcely be justified unless the limitations were outlined, as well as some of the possibilities inherent in the method.

In the first place, it is obvious that the apparatus in its present stage is limited to the separation and study of small amounts of material, probably not more than 0.5 gm. of material or less. On the other hand, there are no lower limitations on the amount of substance which may be studied and separated, if a sufficiently sensitive method of estimation, such as fluorometric, spectroscopic, color test, biological assay, etc., is at hand. A total amount of material which is of the order of 100-fold or more of the limiting sensitivity of the test is sufficient. With our present methods of estimation, this amount would be of the order of a few micrograms of substance for many important drugs.

Secondly, the method is restricted in its use to substances which show appreciable solubility in two or more immiscible solvents.

Thirdly, it is to be expected that the method will become very much involved as the complexity of the mixture increases. The specificity of certain solvents and combinations of solvents will then become of very great importance and may well offer considerable hope of success.

With the opportunity for further study, still other limitations may present themselves. This is also true of the inherent possibilities, of which the following, among others, are apparent thus far.

1. The substances under study are not subject to drastic conditions, such as the high temperatures of distillation, exposure to strong chemical reagents, etc. The method is, therefore, especially suitable for studying labile compounds which are easily altered in a chemical way.

2. The simplicity of the apparatus and the close approach to theoretical performance permit accurate distribution curves to be estimated and quantitative relationships to be established at any point desired in the work. The method lends itself particularly to quantitative interpretation.

3. From the rate of migration of a substance in the apparatus, its distribution constant may be calculated. It is thus possible to characterize an unknown substance, never before isolated, in terms of its distribution constants in mixtures of different solvents. To accomplish this, a sufficiently sensitive method of estimation, such as color test, spectroscopic estimation, etc., is the only requisite. The information so obtained can then lead directly to isolation.

4. The method offers many possibilities for establishing a practical degree of homogeneity with extremely small amounts of material, because

of the fact that a single substance, migrating independently, always should give a distribution curve in agreement with a calculated one.   In this way, the method offers possibilities for establishing specificity for many of the colorimetric and spectroscopic estimations so commonly used in biochemistry.

These suggestions by no means exhaust all the possibilities.   It is our intention to exploit to the fullest extent such possibilities and, at the same time, to try to improve on the mechanical aspects of the apparatus.   At the present time, plans are being made for the construction of a similar apparatus from glass.   Improvements can also be made in the method of equilibration and manipulation.

SUMMARY

A method and apparatus for the precise study of the distribution characteristics of small amounts of organic compounds between two liquid phases have been presented.   The method is well suited for fractionation, proof of homogeneity and identification.   A direct relationship of the process to diffusion phenomena has been shown.

BIBLIOGRAPHY

1. Craig, L. C., *J. Biol. Chem.*, **150**, 33 (1943).
2. Cornish, R. E., Archibald, R. C., Murphy, E. A , and Evans, H. M , *Ind. and Eng. Chem.*, **26**, 397 (1943).
3. Varteressian, K A., and Fenske, M. R., *Ind and Eng. Chem.*, **28**, 1353 (1936).
4. Ney, W. O., and Lochte, H. L., *Ind. and Eng. Chem.*, **33**, 825 (1941).
5. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 91 (1941).
6. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 1358 (1941).
7. Wiener, O., *Ann. Phys. u. Chem.*, **49**, 105 (1893).

TRANSFORMATIONS OF AN ACYL DIKETOPIPERAZINE

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The experiments reported in this communication concern the synthesis and the transformations of a peptide derivative containing a diacylated amino acid residue corresponding to the general structure

$$\begin{matrix} R \cdot CO & \\ & \diagdown \\ & N \cdot R'' \\ & \diagup \\ R' \cdot CO & \end{matrix}$$
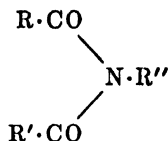
The study of synthetic compounds of this type was undertaken in order to learn whether the presence of similar structural groupings in proteins must be considered.

In the present experiments the unsaturated peptide acetyldehydrophenylalanyl-*l*-proline (I) (1) is used as the starting material. On treatment with acetic anhydride at room temperature, 1 molecule of water is split off and the optically active N-acetyldehydrophenylalanyl-*l*-proline diketopiperazine (II) is formed.

$$\begin{matrix} & CH \cdot C_6H_5 & \\ & \| & \\ CH_3 \cdot CO{-}NH \cdot C \cdot CO{-}N & \\ & \diagup \quad \diagdown \\ HOOC \cdot CH \quad \quad CH_2 \\ | \quad \quad \quad | \\ CH_2{-}{-}{-}CH_2 \end{matrix}$$

(I)

$$\begin{matrix} & CH \cdot C_6H_5 & \\ & \| & \\ & C{-}{-}CO & \\ CH_3 \cdot CO \xrightarrow{(C)} N \quad \quad N & (B) \\ (A) \diagdown CO{-}CH \quad CH_2 \\ | \quad \quad | \\ CH_2{-}{-}CH_2 \end{matrix}$$

(II)

$$\begin{matrix} & CH \cdot C_6H_5 & \\ & \| & \\ CH_3 \cdot CO{-}NH \cdot C \cdot COOH \end{matrix}$$

(III)

$$\begin{matrix} & CH \cdot C_6H_5 & \\ & \| & \\ CH_3 \cdot CO{-}NH \cdot C \cdot CO{-}N & \\ & \diagup \quad \diagdown \\ NH_2 \cdot CO \cdot CH \quad \quad CH_2 \\ | \quad \quad \quad | \\ CH_2{-}{-}CH_2 \end{matrix}$$

(IV)

535
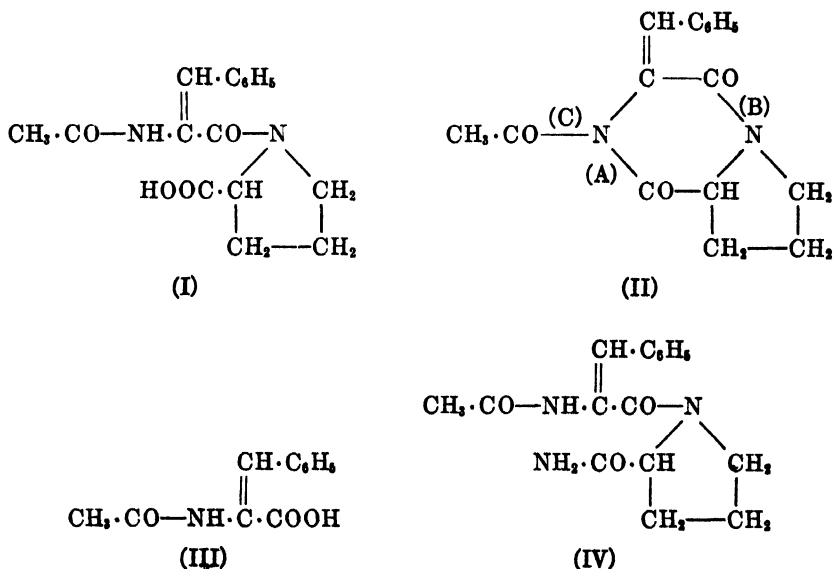
Whereas diketopiperazines are, in general, rather stable, Compound II is quite reactive. It is racemized in contact with an aqueous solution of sodium acetate at room temperature. At steam bath temperature it is racemized and then hydrolyzed to form the sodium salt of an inactive acid, $C_{16}H_{18}O_4N_2$, melting at 223–225°. That this acid is not acetyl-*dl*-prolyl-dehydrophenylalanine, but rather acetyldehydrophenylalanyl-*dl*-proline, follows from the fact that on mild hydrolysis with hydrochloric acid acetyl-dehydrophenylalanine (acetaminocinnamic acid) (III) is obtained. The latter substance is also produced on hydrolysis of acetyldehydrophenylalanyl-*l*-proline. In contrast to the optically active diketopiperazine (II), the peptide acetyldehydrophenylalanyl-*l*-proline is not appreciably racemized when heated with sodium acetate solution at 90° for 2 hours.

When diketopiperazine (II) is treated with ammonia, its heterocycle is

(V)   (VI)

(Va)   (VII)

(Vb)   (VIII)
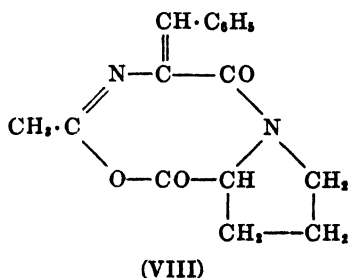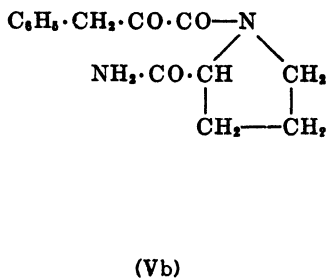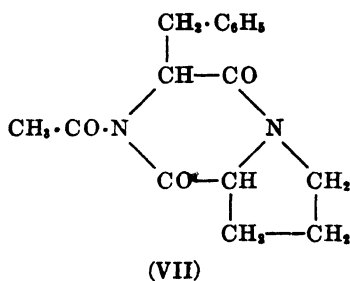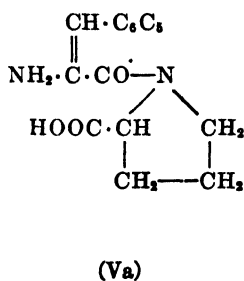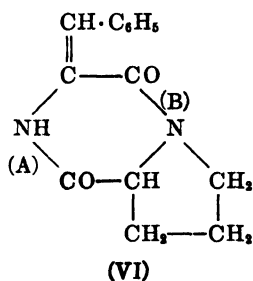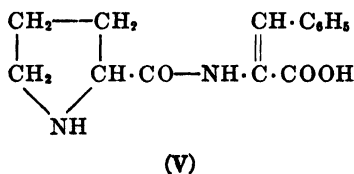
opened and a dipeptidamide is formed. Thus, the *l* form of Compound II yields as the main product acetyldehydrophenylalanyl-*l*-prolinamide (IV).[1] As a by-product, a small amount of *dl*-prolyldehydrophenylalanine (V) is obtained.

When the *l*-diketopiperazine (II) is added to an ethereal solution of glycine ester or to an aqueous solution of the sodium salt of glycine, the acetyl group of Compound II is split off and dehydrophenylalanyl-*l*-proline diketopiperazine (VI) results. In a similar experiment, the *l*-diketopiperazine (II) was added to an aqueous solution of the sodium salt of *l*-leucine. Here, acetyl-*l*-leucine and *dl*-prolyldehydrophenylalanine (V) were isolated.

On catalytic hydrogenation in the presence of palladium black, the *l*-diketopiperazine (II) takes up 1 mole equivalent of hydrogen. N-Acetyl-*l*-phenylalanyl-*l*-proline diketopiperazine (VII) was isolated as one of the reaction products, in a yield of about 50 per cent.

The catalytic hydrogenation of *dl*-prolyldehydrophenylalanine (V) in aqueous acetone results in the formation of small amounts of an inactive, hydrated prolylphenylalanine diketopiperazine, melting at 73–75°, besides larger amounts of an inactive, anhydrous prolylphenylalanine diketopiperazine, melting at 130–132°.

Upon hydrogenation of dehydrophenylalanyl-*l*-proline diketopiperazine (VI), the diketopiperazine of *l*-phenylalanyl-*l*-proline was isolated. When this diketopiperazine was hydrolyzed by boiling for 5 hours with 10 equivalents of 3 N HCl, pure *l*-prolyl-*l*-phenylalanine could be isolated in 25 per cent yield. This peptide was identical with a peptide obtained by Osborne and Clapp (2) on protracted boiling of gliadin with 25 per cent sulfuric acid. Fischer and Luniak (3) have shown that this substance is *l*-prolyl-*l*-phenylalanine.

The synthesis and the properties of the following proline derivatives are reported in this communication: acetyldehydrophenylalanyl-*l*-proline diketopiperazine (and its *dl* form), acetyldehydrophenylalanyl-*l*-prolinamide (and its *dl* form), acetyldehydrophenylalanyl-*dl*-proline, *dl*-prolyldehydrophenylalanine, dehydrophenylalanyl-*l*-proline diketopiperazine, acetyl-*d*-phenylalanyl-*l*-prolinamide, *l*-phenylalanyl-*l*-proline diketopiperazine, acetyl-*l*-phenylalanyl-*l*-proline diketopiperazine, *l*-prolyl-*l*-phenylalanine, optically inactive prolylphenylalanine diketopiperazine (two forms).

DISCUSSION

The inactive dipeptide, formed during the interaction of the *l*-diketopiperazine (II) and the sodium salt of leucine, has been formulated as

[1] Similarly, the *dl* form of (II) yields the *dl* form of (IV).

dl-prolyldehydrophenylalanine (V). The alternate structures, dehydro-phenylalanyl-*dl*-proline (Va) and phenylpyruvoyl-*dl*-prolinamide (Vb), are excluded. Compound Va should dissociate into ammonia and phenyl-pyruvoyl-*dl*-proline, while our dipeptide is completely stable. Compound Vb has no carboxyl group, while our dipeptide can be titrated with alkali in 90 per cent alcohol with thymolphthalein. In addition, our dipeptide contains no amino nitrogen.

The peptide anhydride formed by the action of acetic anhydride upon Compound I has been interpreted as an acetylated diketopiperazine of structure (II). Formula VIII, representing an azlactone with an 8-membered ring, was also considered but dismissed, since it could hardly explain the easy formation of prolyldehydrophenylalanine (V).

From the previous description it will be apparent that the interaction of the diketopiperazines (II) and (VI) with basic substances is a rather complex process. This is due to the presence in these diketopiperazines of several groupings which are sensitive to basic reagents. The acetyl-*l*-diketopiperazine (II), for instance, contains three CO—N linkages and an asymmetric carbon atom which is easily racemized, while the acetyl-free diketopiperazine (VI) contains two CO—N linkages and an asymmetric carbon atom. The CO—N linkages are marked in Formulas II and VI by the letters A, B, and C. It is a noteworthy fact that the action of the various basic reagents upon the base-sensitive groupings of Formula II (or VI) differs widely not only in regard to the final product of the reaction, but also with respect to the initial point of attack. One possible explanation is offered by the hypothesis that each of the basic reagents combines with Compound II˙ in a highly specific manner, just as each of several heterospecific enzymes may combine with the same substrate in a specific manner (4). In both cases, then, the structure of the intermediate addition compound modifies the hydrolytic susceptibility of the CO—N linkages present and thus determines the course of the reaction.

As an illustration, the reaction between Compound II and the amine groups of ammonia, sodium leucinate, sodium glycinate, and glycine ester may be discussed. The interaction of the acetyl-*l*-diketopiperazine (II) with sodium leucinate, which produces *dl*-prolyldehydrophenylalanine and acetylleucine, must consist of three distinct processes. The asymmetric carbon atom is racemized and the acetyl group of diketopiperazine (II) migrates to the amino group of sodium leucinate by a process of trans-acetylation. The order in which these two reactions occur is not known, but no matter what it is, the over-all result must be the *dl* form of the acetyl-free diketopiperazine (VI). Subsequently, the ring of Compound VI is opened hydrolytically at B, yielding *dl*-prolyldehydrophenylalanine.

The interaction of sodium glycinate and the acetyl-*l*-diketopiperazine

(II) results mainly in a deacetylation of Compound II, probably by trans-acetylation. The resulting diketopiperazine, which can be isolated in 80 to 90 per cent yield, is only slightly racemized, its specific rotation being 2.5 per cent lower than that of the diketopiperazine (VI) resulting from the interaction of Compound II with glycine ester in anhydrous ethereal solution. In the latter case, racemization seems unlikely.

When diketopiperazine (II) reacts in aqueous pyridine with an excess of ammonia, the main process is the opening of linkage A with the formation of acetyldehydrophenylalanyl-*l*-prolinamide. Racemization occurs to a small extent, as is indicated by the occasional isolation of the *dl*-amide. The train of reactions, previously discussed for sodium leucinate, also occurs to a slight extent with ammonia, as shown by the formation of *dl*-prolyl-dehydrophenylalanine. Still another transformation of diketopiperazine (II), its racemization by aqueous sodium acetate and subsequent hydrolysis, has already been mentioned.

From what has been said before, it will be noted that the ring system of the acetylated diketopiperazine (II) is opened by ammonia at linkage A. If, however, the attack on the diketopiperazine ring is preceded by the removal of the acetyl group, as in the action of sodium leucinate on Compound II to form VI, then the diketopiperazine ring is opened mainly at B. It appears, therefore, that the comparative stability of the two CO—N linkages A and B towards bases depends upon the presence or absence of the acetyl group in Compounds II and VI respectively. This statement, derived from the action of amines upon the diketopiperazines, holds also for the action of sodium hydroxide. When Compound II is treated with 1 equivalent of sodium hydroxide, acetyldehydrophenylalanyl-*dl*-proline is formed, while from the *l* form of Compound VI *dl*-prolyldehydrophenyl-alanine is obtained.

In regard to transacetylation, it may be pointed out that the formation of acetyl-*l*-leucine during the interaction of the acetylated diketopiperazine (II) and the sodium salt of leucine is not a new phenomenon, but is analogous to the acyl migrations reported in previous communications (5).

Easy racemization of amino acids or their derivatives has hitherto been observed under various conditions. Cyclic amino acid derivatives such as hydantoins and diketopiperazines are racemized in slightly alkaline aqueous solution (6–9). Active acetylated amino acids become inactivated by the action of acetic anhydride or azlactones in non-aqueous solution (10, 11) or in alkaline aqueous solution under the influence of benzoyl chloride, acetic anhydride, or ketene (12–14). It is generally believed that the mechanism of the alkaline racemization of the hydantoins and diketo-piperazines is different from that occurring during the action of acetic anhydride (15). This is in good agreement with the observation that the

active diketopiperazine (II) is formed in the presence of an excess of acetic anhydride without any appreciable racemization, while the same active diketopiperazine is racemized by sodium acetate in aqueous solution under mild experimental conditions.

It has been reported in the literature that l-prolyl-l-proline (16) and glycyl-l-proline and glycyl-l-hydroxyproline (17) show a definite tendency towards ring closure, resulting in the formation of diketopiperazines. In these examples the carboxyl group of proline or hydroxyproline participates in the ring closure. The same is the case when diketopiperazine (II) is formed from the open peptide (I) under the influence of acetic anhydride. In the formation of a diketopiperazine during the catalytic hydrogenation of prolyldehydrophenylalanine, which is reported in this communication, the imino group of proline forms the ring. All these examples seem to indicate that the presence of proline or hydroxyproline in a dipeptide favors diketopiperazine formation, possibly for stereochemical reasons.

The authors wish to thank Dr. A. Elek, who performed the elementary analyses reported in the experimental section, for his valuable assistance.

<center>EXPERIMENTAL</center>

Acetyldehydrophenylalanyl-l-proline Diketopiperazine (*II*)—30 gm. of acetyldehydrophenylalanyl-l-proline were mixed with 4 gm. of anhydrous sodium acetate and covered with 80 cc. of acetic anhydride. On shaking, a colorless solution resulted, from which the acetyldiketopiperazine crystallized as colorless needles within 2 hours. The following day the excess acetic anhydride was decomposed at 0° by the addition of a mixture of 140 cc. of 10 per cent HCl and 300 gm. of ice. The diketopiperazine was dried *in vacuo* over P_2O_5 and NaOH. Yield, 23 gm. M.p., 168–170°. After one recrystallization from 200 cc. of acetone by the addition of 300 cc. of water, the yield was 20 gm., or 70 per cent of the theory. M.p., 172–173°.

<center>

$C_{16}H_{16}O_3N_2$.   Calculated.   C 67.6, H 5.7, N 9.8
284 3   Found   " 67.6, " 5.7, " 9.9

</center>

$[\alpha]_D^{25}$ = +48.8° (5 per cent, in pyridine). After another recrystallization, $[\alpha]_D^{25}$ = +49.3°.

When the acetyldiketopiperazine was prepared without the use of sodium acetate, it took somewhat longer to obtain a clear solution and no crystallization occurred. After 24 hours, the diketopiperazine was isolated by the addition of ice and diluted HCl as previously described. The yield was 68 per cent of the theory. M.p., 172–173°

<center>

Found, C 67.6, H 5.6, N 9.8
$[\alpha]_D^{25}$ = + 48.5° (5%, in pyridine)

</center>

Acetyldehydrophenylalanyl-dl-proline—10 gm. of acetyldehydrophenyl-alanyl-*l*-proline diketopiperazine were heated on the steam bath with a solution of 5 gm. of sodium acetate in 50 cc. of water. After about 2 hours, a clear solution resulted which after another hour's heating and on acidification with HCl, deposited 9.5 gm. of crude acetyldehydrophenyl-alanyl-*dl*-proline, melting at 212–214°. After two recrystallizations from methanol by the addition of water, 5.5 gm., melting at 223–225° (with decomposition), were obtained. The crude material contained a small amount of an optically active substance which was removed during recrystallization.

$C_{16}H_{18}O_4N_2$. Calculated C 63.6, H 6.0, N 9.3
302.3 Found. " 63 5, " 5 9, " 9.4

On hydrolysis with a 1:1 mixture of acetic acid and 3 N HCl, the substance yielded acetaminocinnamic acid and *dl*-proline.

28.4 gm. of acetyldehydrophenylalanyl-*l*-proline diketopiperazine were suspended in 100 cc. of acetone, the mixture cooled, and 100 cc. of N NaOH added slowly with vigorous stirring. The initial bright red color soon disappeared. After 10 minutes the yellow solution was filtered, 100 cc. of water were added, and the solution was acidified to Congo red by the addition of 105 cc. of N HCl. Crystallization began almost immediately. The yield of crude material was about 15 gm., melting at 200–206° (with decomposition). After several recrystallizations from methanol, 2.3 gm. of acetyldehydrophenylalanyl-*dl*-proline were obtained, m.p. 224–225° (with decomposition). A mixture of this substance with that obtained by the sodium acetate procedure showed no depression of the melting point.

Found, C 63.3, H 5.9, N 9.2

In a 1 dm. tube, a 5 per cent solution of the material in pyridine exhibited no optical activity.

Acetyldehydrophenylalanyl-dl-proline Diketopiperazine—1 gm. of acetyl-dehydrophenylalanyl-*dl*-proline was treated at 37° with 2.5 cc. of acetic anhydride in the presence of 0.12 gm. of anhydrous sodium acetate. After 24 hours, decomposition with ice and dilute HCl yielded 0.7 gm. of crude *dl*-diketopiperazine. One recrystallization from acetone with the addition of water yielded 0.45 gm., melting at 149–151°.

$C_{16}H_{16}O_3N_2$. Calculated. C 67.6, H 5.7, N 9.8
284.3 Found. " 67.3, " 5 5, " 9.7

The pyridine solution was optically inactive. The same substance may be obtained by the action of sodium acetate on the acetyl-*l*-diketopiperazine at room temperature according to the following procedure.

5 gm. of acetyldehydrophenylalanyl-*l*-proline diketopiperazine and 5 gm.

of hydrated sodium acetate were dissolved in a mixture of 100 cc. of acetone and 150 cc. of water. After 24 hours, the solution was concentrated *in vacuo* and the resulting precipitate recrystallized from absolute ethanol, and subsequently from acetone with the addition of water. 2.0 gm. were obtained, melting at 148–151°. This substance was identical with that described earlier in this section.

<div align="center">Found, C 67 4, H 5.8, N 9.9</div>

In a 2 dm. tube, a 5 per cent solution of this substance in pyridine showed no optical activity.

Acetyldehydrophenylalanyl-l-prolinamide—To a thoroughly chilled and agitated mixture of 150 cc. of concentrated ammonia and 60 cc. of pyridine were slowly added 30 gm. of acetyldehydrophenylalanyl-*l*-proline diketopiperazine. Solution of the diketopiperazine was followed by crystallization of the rectangular prisms of the amide. The mixture was kept in the cold room overnight and then filtered.[2] The yield of crude acetyldehydrophenylalanyl-*l*-prolinamide, melting at 231–235°, was 25 gm. After recrystallization from methanol, in which the amide is quite soluble, 6 gm., melting at 239–241°, were obtained.

<div align="center">

$C_{16}H_{19}O_3N_3$.   Calculated.   C 63.5, H 6.4, N 13.95

301.3   Found.   " 63.5, " 6.4, " 13.85

</div>

$[\alpha]_D^{26}$ = −105.7° (2 per cent in pyridine, 2 dm. tube). After another recrystallization $[\alpha]_D^{24}$ = −104.0°.

In the preparation of this *l*-amide, there were isolated in one instance 7.6 gm. of an isomeric acetyldehydrophenylalanylprolinamide. For recrystallization it was dissolved in a boiling mixture of 135 cc. of acetic acid and 5 cc. of water and precipitated by the subsequent addition of 300 cc. of water. Yield, 6 gm., melting at 262–264° (with decomposition). The presence of optical activity could not be determined, since no proper solvent for the amide has been found. This substance may tentatively be regarded as acetyldehydrophenylalanyl-*dl*-prolinamide.

<div align="center">Found, C 63 5, H 6.2, N 14.0</div>

An acetyldehydrophenylalanyl-*dl*-prolinamide which melted at 265° and was apparently identical with the foregoing amide, was obtained when acetyldehydrophenylalanyl-*dl*-proline diketopiperazine was treated with ammonia and pyridine.

<div align="center">Found, C 63.6, H 6.4, N 13.9</div>

Reaction of l-Diketopiperazine (II) with Sodium Leucinate—To a solution of 13.1 gm. of *l*-leucine in a mixture of 100 cc. of acetone and 100 cc. of N

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[2] The filtrate, on acidification with HCl, yielded 1.2 gm. of crude *dl*-prolyldehydrophenylalanine, which after recrystallization melted at 169–171° and was optically inactive. Found, C 64.5, H 6.3, N 10.8.

NaOH were added, with vigorous stirring, 28.4 gm. of acetyldehydrophenyl-alanyl-*l*-proline diketopiperazine. The entire amount was added as one portion. A clear solution resulted with the spontaneous generation of some heat and within a few minutes colorless crystals of *dl*-prolyldehydrophenyl-alanine appeared. After 3 hours, the crystals were removed. Yield, 19 gm. On recrystallization from 250 cc. of 90 per cent methanol, 15 gm. of colorless needles were obtained. For analysis, the peptide was once more recrystallized. M.p., 171–172°. The peptide contained no amino nitrogen.

$C_{14}H_{16}O_3N_2$.   Calculated.   C 64.6, H 6.2, N 10.8
260.3   Found.   " 64.5, " 6.1, " 10.8

A 5 per cent solution in pyridine showed no appreciable optical activity. With N NaOH, *dl*-prolyldehydrophenylalanine forms a difficultly soluble sodium salt from which the free peptide may be regenerated by acidification with HCl.

From the mother liquor of the prolyldehydrophenylalanine there was isolated about 0.4 gm. of unchanged leucine and 5.8 gm. of acetyl-*l*-leucine, melting at 189–190°.

$C_8H_{15}O_3N$.   Calculated.   C 55.5, H 8.7, N 8.1
173.2   Found.   " 55 4, " 8.7, " 8.1
$[\alpha]_D^{24} = -40.2°$ (5%, in pyridine); $[\alpha]_D^{24} = -23 1°$ (3.3%, in ethanol)

Martin and Synge (18) have reported melting points of 182° and of 180–181°. They found for a 3.3 per cent solution in ethanol $[\alpha]_D^{24} = -23.5°$.

Reaction of l-Diketopiperazine (II) with Glycine Ester—14 gm. of acetyl-*l*-diketopiperazine (II) were added at 0° to a solution of 12.5 gm. of glycine ethyl ester in 100 cc. of ether. When the mixture was stirred at room temperature, it generated heat and simultaneously the crystals of the starting material disappeared and the prismatic forms of dehydrophenylalanyl-*l*-proline diketopiperazine appeared. Yield, after 1 hour, 10.1 gm. The substance was recrystallized from absolute ethanol. M.p., 177–180°.

$C_{14}H_{14}O_2N_2$.   Calculated   C 69.4, H 5.8, N 11.6
242.3   Found   " 69 5, " 5 7, " 11.4
$[\alpha]_D^{25 5} = +284.7°$ (5 per cent, in pyridine). After another recrystallization, $[\alpha]_D^{25} = +284.3°$ was found.

It is not known whether this value represents the maximum rotation of the optically pure dehydrophenylalanyl-*l*-proline diketopiperazine.

Reaction of l-Diketopiperazine (II) with Sodium Glycinate—To a solution of 7.5 gm. of glycine in 75 cc. of acetone and 100 cc. of N NaOH, were added 28.4 gm. of acetyldehydrophenylalanyl-*l*-proline diketopiperazine. There was some spontaneous generation of heat, solution occurred, and, after a few minutes, crystallization began. The yield of crude material, melting at 176–177°, ranged from 18.7 to 22.5 gm. After recrystallization from acetone and water and, subsequently, from absolute ethanol, about

15 gm. of dehydrophenylalanyl-*l*-proline diketopiperazine were obtained. M.p., 176–178°. The melting point of a mixture of this product with that of the diketopiperazine described in the preceding section was 176–179°.

Found, C 69.6, H 5.9, N 11.7

$[\alpha]_D^{24}$ = +277 1° (5 per cent, in pyridine). After recrystallization, $[\alpha]_D^{22.5}$ = +277.2°

Apparently the substance was racemized to a slight extent.

Hydrogenation of Dehydrophenylalanyl-l-proline Diketopiperazine—A suspension of 12.1 gm. of dehydrophenylalanyl-*l*-proline diketopiperazine in 200 cc. of ethanol was hydrogenated in the presence of palladium black, and the resulting clear solution evaporated *in vacuo*. The 9.5 gm. of crude material, melting at 131°, were twice recrystallized from absolute ethanol, yielding 5.3 gm. of *l*-phenylalanyl-*l*-proline diketopiperazine. M.p., 135–136°.

$C_{14}H_{16}O_2N_2$ Calculated.   C 68.8, H 6.6, N 11.5
244.3   Found.   " 68 9, " 6.6, " 11.4

$[\alpha]_D^{26}$ = 107 6° and, after recrystallization of the sample, $[\alpha]_D^{26}$ = −106 1°.

3.7 gm. of the saturated diketopiperazine were hydrolyzed by refluxing for 5 hours with 10 per cent HCl. Ammonium acetate was added to the cooled solution until 1.7 gm. of a precipitate appeared. This was recrystallized from 340 cc. of boiling water and yielded 1.1 gm. of *l*-prolyl-*l*-phenylalanine as fine plates, melting at 250–251°. The presence of 1 water of crystallization per molecule of material was demonstrated by drying *in vacuo* at 100°.

$C_{14}H_{18}O_3N_2 \cdot H_2O$. Calculated   C 60.0, H 7.2, N 10.0, H_2O 6.4
280.3   Found.   " 59 6, " 7 2, " 10.0, " 6 2

$[\alpha]_D^{24}$ = −21 2° (5 per cent, in glacial acetic acid) and, after recrystallization of the sample, $[\alpha]_D^{27}$ = −20.6°

$$[\alpha]_D^{26} = -40.8° \ (5\%, \text{ in } 20\% \text{ HCl})$$

Osborne and Clapp reported for their peptide, obtained from gliadin, $[\alpha]_D^{20}$ = −40.9° and −41.55°, while Fischer and Luniak found $[\alpha]_D^{20}$ = −40.9° for their synthetic *l*-prolyl-*l*-phenylalanine.

Dehydrophenylalanine-l-proline Diketopiperazine and NaOH—6.05 gm. of dehydrophenylalanine-*l*-proline diketopiperazine were dissolved in 25 cc. of acetone and 25 cc. of N NaOH were added. Within a short time a sodium salt crystallized which was decomposed by the addition of 75 cc. of water and 26 cc. of N HCl. The crystalline precipitate now formed weighed 6 gm. It was recrystallized several times from acetone-water (5:1) by the addition of more water, yielding 5.0 gm. of *dl*-prolyldehydrophenylalanine, melting at 168–171°.

$C_{14}H_{16}O_3N_2$. Calculated. C 64.6, H 6.2, N 10.8
260.3   Found.   " 64.7, " 6.3, " 11.0

In a 1 dm. tube, a 5 per cent solution of the substance in pyridine exhibited no appreciable optical activity.

Acetyl-d-phenylalanyl-l-prolinamide—When a suspension of 10 gm. of acetyldehydrophenylalanyl-*l*-prolinamide in 80 cc. of absolute methanol was hydrogenated in the presence of palladium black, a clear solution resulted and slightly more than 1 mole equivalent of hydrogen gas was taken up. The clear filtrate was concentrated *in vacuo*. Approximately 7 gm. of acetyl-*d*-phenylalanyl-*l*-prolinamide were obtained, melting at 116–122°. After recrystallization from methanol, the melting point was 120°.

$C_{16}H_{21}O_3N_3 \cdot \frac{1}{2}H_2O$ Calculated. C 61 5, H 7.1, N 13.4, H_2O 3.5
312 4   Found   " 61 5, " 7 1, " 13.5, " 3.9
$[\alpha]_D^{24} = -118\ 8°$ (5 per cent, in glacial acetic acid). After recrystallization, $[\alpha]_D^{23.5} = -118\ 1°$.

Hydrolysis of 3.4 gm. of the acetyl-*d*-phenylalanyl-*l*-prolinamide with concentrated HCl for 9 hours resulted in the direct crystallization from the cooled, acid solution of 1.9 gm. of *d*-phenylalanine hydrochloride. From this, 0.9 gm. of *d*-phenylalanine was obtained.

Calculated, N 8.5; found, N 8.7
$[\alpha]_D^{26} = +34\ 2°$ (2%, in water)

Fischer and Schoeller reported for *d*-phenylalanine $[\alpha]_D^{20} = +35.1°$ (19).

Hydrogenation of dl-Prolyldehydrophenylalanine—A suspension of 13 gm. of *dl*-prolyldehydrophenylalanine in 150 cc. of a 6:1 acetone-water mixture was hydrogenated over palladium black catalyst in the usual manner, and 1 mole equivalent of hydrogen was taken up. The product was isolated by evaporation *in vacuo*, and the subsequent addition of 50 cc. of water. Two recrystallizations from absolute alcohol yielded 8 gm. of an optically inactive prolylphenylalanine diketopiperazine, melting at 130–132°.

$C_{14}H_{16}O_2N_2$. Calculated. C 68.8, H 6.6, N 11.5
244 3   Found   " 69.0, " 6.6, " 11.3

As a by-product of this hydrogenation, there was also isolated about 1 gm. of a hydrated diketopiperazine, which, on recrystallization from water, appeared as well formed prisms, melting at 73–75°.

$C_{14}H_{16}O_2N_2 \cdot H_2O$. Calculated C 64 0, H 6.9, N 10 5, H_2O 6 9
262 3   Found   " 64.1, " 6.9, " 10 7, " 6.8

Acetyl-l-phenylalanyl-l-proline Diketopiperazine—A suspension of 28.4 gm. of acetyldehydrophenylalanyl-*l*-proline diketopiperazine in 350 cc. of acetone was hydrogenated over palladium black catalyst in the usual manner, and a slight excess over 1 mole equivalent of hydrogen was taken up. About 20 gm. of crude product were isolated by evaporation *in vacuo*, and, after several recrystallizations from methanol by the addition of water,

about 15 gm. of acetyl-*l*-phenylalanyl-*l*-proline diketopiperazine were obtained, melting at 123–125°.

$C_{16}H_{18}O_3N_2$. Calculated. C 67.1, H 6.3, N 9.8
286 3 Found. " 67.1, " 6.6, " 9.8
$[\alpha]_D^{25}$ = +202.6° (5 per cent, in pyridine). After another recrystallization $[\alpha]_D^{24}$ = +201.3°.

Of this material, 5 gm. were refluxed with 35 cc. of concentrated HCl, for 6 hours. Long needles of *l*-phenylalanine hydrochloride were obtained when the solution was cooled. The free *l*-phenylalanine was obtained by treatment with ammonium acetate.

$$[\alpha]_D^{27} = -33.9° \text{ (in water)}$$

BIBLIOGRAPHY

1. Behrens, O. K., Doherty, D. G., and Bergmann, M., *J. Biol. Chem.*, **136**, 61 (1940).
2. Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, **18**, 123 (1907).
3. Fischer, E., and Luniak, A., *Ber. chem. Ges.*, **42**, 4752 (1909).
4. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **138**, 249 (1941)
5. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **175**, 145 (1928). Bergmann, M., du Vigneaud, V., and Zervas, L , *Ber. chem. Ges.*, **62**, 1909 (1929).
6. Dakin, H. D., *Am. Chem. J.*, **44**, 48 (1910).
7. Bovarnick, M., and Clarke, H. T., *J. Am. Chem. Soc.*, **60**, 2426 (1938).
8. Levene, P. A., and Pfaltz, M. H , *J. Biol. Chem.*, **63**, 661 (1925).
9. Bergmann, M., Zervas, L., and Koester, H., *Ber. chem. Ges.*, **62**, 1901 (1929).
10. Bergmann, M., and Zervas, L., *Biochem. Z.*, **203**, 280 (1928).
11. Carter, H. E., Handler, P., and Stevens, C M , *J. Biol. Chem* , **138**, 619 (1941).
12. Fischer, E., *Ber. chem. Ges.*, **32**, 2451 (p. 2464) (1899).
13. du Vigneaud, V., and Sealock, R. R., *J. Biol. Chem.*, **96**, 511 (1932). du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98**, 295 (1932).
14. Cahill, W. M., and Burton, I. F., *J. Biol. Chem.*, **132**, 161 (1940). Jackson, R. W., and Cahill, W. M., *J. Biol. Chem.*, **126**, 37 (1938).
15. Clarke, H. T., in Gilman, H., Organic chemistry, New York, 2nd edition, **2** (1943).
16. Abderhalden, E., and Nienburg, H., *Fermentforschung*, **13**, 573 (1933).
17. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153**, 627 (1944).
18. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 91 (1941). Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 313 (1943).
19. Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 1 (1907).

AMINO ACIDS OF ISINGLASS

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The use of isinglass in the treatment of certain conditions for which a blood substitute is required has been investigated by Taylor and his associates (1, 2). The encouraging results obtained by these workers have occasioned much interest in the composition of this fish protein. It is well known that, in a number of circumstances in which blood substitutes may be used, a marked negative nitrogen balance ensues. This raises the question as to whether or not isinglass constitutes a good source of readily available essential amino acids for the synthesis of body protein, since in the absence of these metabolites it is impossible to maintain nitrogen balance. A search of the literature failed to reveal any pertinent information. The problem may be attacked by two methods: (1) by analysis of the protein; (2) by studies of growth and nitrogen balance on animals. Investigations by the latter method are being carried out by the workers mentioned above (3). The analytical work was undertaken by the writers at the suggestion of Professor C. H. Best. This report describes the estimation of the various amino acids liberated by the acid hydrolysis of isinglass.

The material used was part of Batch 20 prepared in Professor Taylor's laboratory from the dried swim-bladder of the hake (probably *Urophycis tenuis* or *Urophycis chuss*). Dried swim-bladders obtained commercially were soaked in ice water 24 to 48 hours, and then in ice-cold saturated calcium hydroxide 6 to 12 days, the solution being changed each day. They were then further treated with cold 0.5 per cent hydrochloric acid for 3 to 4 hours and rinsed with cold 1 per cent sodium chloride solution. The acid treatment causes the isinglass to exude as a clear gel and the sodium chloride removes acid and prevents the jelly from taking up much water. The jelly was drained off, heated to 55°, and the residue which settled out was discarded. The solution was made to pH 4.0, heated to 90° for 4 minutes, cooled to 70°, and filtered with the aid of Super-Cel. Sodium bicarbonate was added to pH 6.3 and the isinglass precipitated by running the solution into acetone. The isinglass was redissolved, autoclaved, and reprecipitated in acetone.[1]

All figures are expressed as a percentage of the ash- and moisture-free

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[1] The procedure now being used for the preparation of the isinglass has been simplified considerably from the description given here.

protein. All evaporations were carried out *in vacuo* at 35–50°. The melting point values quoted are corrected.

<div align="center">EXPERIMENTAL</div>

The moisture of the isinglass was estimated by drying it to constant weight *in vacuo* over phosphorus pentoxide; sulfur was determined as sulfate after fusion with sodium peroxide and sodium carbonate. Nitrogen was determined by the micro-Kjeldahl method. An appropriate weight of the protein was digested for 3 hours with concentrated sulfuric acid. The solution was then made up to volume and aliquots were digested and nitrogen determined in the usual way. The proximate analysis, based on ash- and moisture-free material, was 0.632 per cent sulfur and 18.21 per cent nitrogen; the moisture content was 4.52 and the ash 3.85 per cent.

Rate of Liberation of Amino Nitrogen—The advisability of determining the shortest time necessary for maximal hydrolysis of the protein is dictated by the established fact that certain amino acids are destroyed or modified by prolonged boiling with strong mineral acids. The rate of liberation of amino groups was therefore determined by Van Slyke's gasometric method on a sample of isinglass which was refluxed with 8 N hydrochloric acid. The amino nitrogen, measured as per cent of the total nitrogen, was as follows: in 1 hour, 61.6; 2 hours, 69.2; 4 hours, 71.0; 6 hours, 70.7; 8 hours, 71.2; 10 hours, 71.7; 12 hours, 71.4; 24 hours, 72.3.

Apparently isinglass is very quickly hydrolyzed under these conditions, since after 4 hours the liberation of amino nitrogen has reached a definite plateau.

Hydrolysis—68.65 gm. of isinglass (ash- and moisture-free) were hydrolyzed by refluxing with 460 ml. of 8 N hydrochloric acid for 8 hours. The hydrolysate, which was quite clear and very light brown in color, was freed from excess hydrochloric acid by evaporation to a syrup several times. After the final evaporation, the syrup was diluted to about 200 ml. The melanin was filtered off, but the amount obtained was negligible. The solution was then decolorized by boiling it for 5 minutes with 2 gm. of charcoal and was made up to 500 ml. Aliquots were used for the estimation of the amino acids.

Analysis. Tyrosine—A negative Millon's test (4) on both the original material and the products of hydrolysis indicated the absence of this amino acid.

Tryptophane—Tests for tryptophane by the glyoxylic acid method (5) on the unhydrolyzed isinglass were negative. Similar negative results were obtained on samples hydrolyzed with sodium hydroxide.

Phenylalanine—Application of Block and Bolling's modification (6) of the Kapeller-Adler colorimetric method (7) gave a figure of 1.83 per cent.

Arginine—75 ml. of the isinglass hydrolysate (equivalent to 10.3 gm. of protein) were subjected to the diflavianate procedure described by Vickery (8). 2.69 gm. of recrystallized arginine flavianate were obtained. The product gave a satisfactory decomposition point of 259–261° (accepted value, 258–260°); yield, 9.33 per cent. Although further crops of flavianate were isolated from the filtrate, none of them proved to be the arginine derivative.

Proline—The mother liquor from the determination of arginine was evaporated *in vacuo* to about 100 ml. Proline was precipitated according to the method of Bergmann (9) with ammonium rhodanilate; yield, 9.5 gm. The yield after application of Bergmann's correction for solubility was 17.8 per cent. A sample of the product was recrystallized (recovery, 85 per cent) and analyzed.

$(C_{16}H_{14}N_6S_4Cr) \cdot (C_6H_{10}O_2N) \cdot H_2O$. Calculated: C 41.7, H 4.3, N 16.2
Found. " 41.8, " 4.5, " 16.4

Hydroxyproline—The filtrate from the precipitation of proline rhodanilate was evaporated to about 80 to 90 ml. and treated with ammonium reineckate (10, 9). Three crops of reineckate were collected, combined, and decomposed (9). After evaporation of the hydroxyproline solution to dryness *in vacuo*, the residue did not redissolve completely in the expected manner. A yellow substance consisting mostly of needles was insoluble in the 75 ml. of water used; weight, 0.62 gm. A similar residue weighing 0.1 gm. was obtained by again evaporating the supernatant solution to dryness and taking up the residue in water. These crops were not identified. The final hydroxyproline fraction weighed 0.399 gm. and contained 11.4 per cent nitrogen (calculated, 10.69). The most likely contaminant would be ammonium salts. Nessler's test for ammonia was positive and quantitative determination by distillation from calcium oxide revealed the presence of 1.86 per cent nitrogen in the form of ammonium salts. Assuming that the contaminant was ammonium acetate, the residue would then have a nitrogen content of 10.63 per cent.[2] Application of Bergmann's correction based on a recovery experiment gives 4.68 per cent hydroxyproline in isinglass. It is believed that this figure is a minimal one and may possibly be considerably lower than the true value.

Glycine—An attempt was made to estimate this amino acid by formation and isolation of the ethyl ester hydrochloride. A 5.00 ml. aliquot of the hydrolyzed protein (0.687 gm.) was taken to absolute dryness and then treated in the usual way to form the ethyl ester hydrochloride. Three crops were obtained: 48.4 mg., m.p. 144–145°; 61.5 mg., m.p. 144–145°; and

[2] Through an unfortunate accident, this fraction was lost before it could be more definitely characterized.

12.0 mg., m.p. 141–143°; accepted m.p. 144°.   This amount of hydrochloride corresponds to a glycine content of 9.55 per cent.   In view of the well known fact that the quantitative separation of glycine as its ethyl ester hydrochloride is often difficult (11), this figure must be considered as a minimal one and may be markedly lower than the actual proportion of glycine in isinglass.

Histidine—An aliquot of the hydrolyzed isinglass solution equivalent to 40 gm. of ash- and moisture-free protein was treated with phosphotungstic acid (12).   The filtrate was set aside for the determination of the dicarboxylic acids.

After decomposition of the phosphotungstate by extraction of the acid mixture with a solution of amyl alcohol, ether, and absolute alcohol, the fraction containing the bases was evaporated *in vacuo* to about 400 ml. and a silver salt precipitation was performed (13).   Although silver sulfate was crystallizing out of the amino acid solution, a positive test for excess silver ion could not be obtained.[3]   The filtrate was set aside for the determination of lysine.

The silver precipitate was freed from inorganic ions in the usual way and the resulting solution was made up to 250 ml.   Histidine was then determined colorimetrically by the Kapeller-Adler method (14) as modified by Conrad and Berg (15) and by Woolley and Peterson (16).   An average value of 1.04 per cent was obtained.   When this figure is corrected for the solubility of histidine phosphotungstate, as recommended by Van Slyke *et al.* (12), the level is raised to 1.09 per cent.   A negative test for histidine was obtained on the lysine fraction, thus indicating the completeness of the precipitation of histidine by the silver salt treatment.

Hydroxylysine—The filtrate from the silver salt precipitation was acidified with sulfuric acid and the barium sulfate filtered off and washed thoroughly.   Silver was precipitated with hydrogen sulfide and the filtrate made up to 100 ml.   Aliquots of 10.0 ml. were treated with 0.5 M periodic acid and the resulting formaldehyde precipitated as the dimedon derivative (17, 18).   An average yield of 25.8 mg. of dimedon derivative was obtained (m.p. 189°; accepted m.p. 189°), corresponding to 0.36 per cent hydroxylysine in isinglass.   Schryver, Buston, and Mukherjee (19) reported the isolation of an "oxylysine" from the isinglass obtained from the swim-bladder of the sturgeon.   They reported figures of 2.98 to 3.3 per cent "oxylysine" in isinglass.   This value was not derived by isolation of crystalline

[3] In an attempt to produce evidence of an excess of silver ion, 1.5 volumes of hot saturated silver sulfate solution were added to the amino acid solution.   A positive test for silver ions was then obtained.   However, on evaporation of the mixture to the desired volume (about 400 ml.) the presence of excess silver ion could no longer be demonstrated despite the fact that silver sulfate was crystallizing from the solution.

derivatives but by one of the following procedures, both of which are based on the insolubility of the barium carbamate of the hydroxyamino acid in cold water.

1. After separation of the barium salts of the dicarboxylic acids, the remaining amino acids are precipitated as barium carbamates. These are extracted with ice-cold water, thus leaving behind the insoluble "oxylysine" and glycine compounds. Decomposition of the residue with hot water and treatment with phosphotungstic acid precipitate the "oxylysine," which is estimated by the total nitrogen in the fraction.

2. The bases are precipitated as the phosphotungstates and then after decomposition are precipitated as barium carbamates. These are extracted with ice water, leaving behind the insoluble "oxylysine" compound. Nitrogen is determined in this residue and taken as a measure of the "oxylysine."

These methods will tend to overestimate rather than underestimate the "oxylysine" in proteins. The large discrepancy between the figure here reported and those given by Schryver et al. may possibly be accounted for by the different sources and methods of preparation of the isinglass. Further, the compound isolated by the English workers may not have the same structure as the one which is determined by the periodate oxidation method.

Lysine—As was pointed out previously, the lysine fraction gave a negative test for histidine; however, a strongly positive test for arginine was obtained. A quantitative colorimetric determination of arginine by Weber's modification (20) of the Sakaguchi reaction indicated the presence of 282 mg., thus revealing the incomplete precipitation of this amino acid as silver salt under the conditions used. An aliquot of the lysine fraction (68 ml. containing an estimated 192 mg. of arginine) was treated with flavianic acid in the usual way and an amount of flavianate equivalent to 186 mg. of the base was isolated. The filtrate, after being freed from flavianic acid and sulfate, still gave a positive test for arginine. Since the presence of some hydroxylysine had been previously demonstrated, it was thought advisable to attempt to obtain a sample of this picrate from the lysine fraction in the manner described by Van Slyke et al. (21). Accordingly, the greater part of the lysine was removed by adding enough picric acid to the filtrate to combine with three-quarters of the amino nitrogen present. The yield of crude lysine picrate was 3.09 gm.; the explosion point, 257°. The derivative was recrystallized from water. The weight of lysine picrate was 2.55 gm. (corrected for solubility of picrate in water 2.80 gm.; explosion point, 262°); yield, 4.21 per cent; corrected for solubility of the phosphotungstate (12), 4.35 per cent.

Addition of more picric acid to separate any hydroxylysine led to the

isolation of two more crops of impure picrate, weighing 0.49 and 0.16 gm. respectively. These were recrystallized separately from water but no homogeneous product could be obtained from the latter crop. From the former 0.19 gm. of unidentified picrate was obtained. Decomposition point, 182–183°. The hydroxylysine picrate described by Van Slyke *et al.* (21) was not isolated.

The mother liquor from the lysine picrate was discarded.

Dicarboxylic Acids—The filtrate from the precipitation of the bases was freed from phosphotungstic acid. The solution was made alkaline to thymolphthalein and the ammonia was removed by evaporation *in vacuo*. The dicarboxylic acids were then twice precipitated as the barium salts according to Dakin's modification (11) of Foreman's method (22). Glutamic acid was isolated as the hydrochloride, and aspartic acid as the copper salt. The weight of the first crop of glutamic acid hydrochloride was 4.08 gm. (N, 7.88; calculated, 7.63 per cent); yield, 8.19 per cent. The filtrate was evaporated and allowed to stand in the refrigerator at 0° for about 4 weeks but no more of the compound precipitated. The solution was freed from excess hydrochloric acid by distillation *in vacuo* and then treated at the boiling point with an excess of copper carbonate. The excess reagent was filtered off while the mixture was still hot and was repeatedly extracted with boiling water. Despite this very thorough washing (the washes appeared colorless) the 15.4 gm. of copper carbonate still contained 30.8 mg. of nitrogen. This has not yet been identified. The washes and filtrate were combined and evaporated to about 150 ml. and placed at 0° overnight. Clusters of fine blue needles typical of copper aspartate separated; weight, 3.45 gm. (air-dried).

$C_4H_5O_4NCu\cdot4H_2O$. Calculated, N 5.25, H_2O 27.0; found, N 5.37, H_2O 27.14
(Dried at 130° for 3 hours *in vacuo* over phosphorus pentoxide)

In an attempt to obtain a second crop of glutamic acid hydrochloride, the filtrate was freed from copper with hydrogen sulfide and then evaporated *in vacuo* to dryness. The residue was covered with about 5 to 10 ml. of concentrated hydrochloric acid and the mixture was warmed until the solid matter dissolved. The fraction was left at 0° for 2 days. The weight of the hydrochloride was 1.29 gm. (per cent N, 11.6). The hydrochloride quite obviously was not that of glutamic acid. Negative tests for ammonia, histidine, and arginine were obtained. Glycine was the most probable contaminant of this fraction (per cent N calculated for hydrochloride, 12.55). The isolation of 0.85 gm. of glycine ethyl ester hydrochloride from the above preparation proved that glycine had been carried down in the dicarboxylic acid fraction (per cent N, 10.01; calculated for glycine ethyl ester hydrochloride, 10.03; m.p. 144–145°; accepted m.p. 144°). Assum-

ing that glycine is the only contaminant, then the crop of hydrochloride isolated would contain 0.26 of the glutamic acid derivative corresponding to 0.52 per cent of the isinglass. The actual separation has not been completed. After hydrolysis of the ester linkages, an attempt was made to isolate glutamic acid from the filtrate from the glycine ethyl ester hydrochloride. A crop of impure hydrochloride was obtained; weight, 0.15 gm. (corrected for aliquots); per cent N, 9.36. No further work was done on this fraction.

The filtrate from the second crop of hydrochloride was evaporated *in vacuo* six times to free it from excess hydrochloric acid and copper salts were then formed in the usual way. The excess copper carbonate which was filtered off again contained nitrogen (8 mg.) which could not be eluted with boiling water. The weight of the copper aspartate was 0.21 gm.; per cent nitrogen, 6.45; water, 12.1. This analysis corresponds to a partly dehydrated derivative of the formula $C_4H_5O_4NCu \cdot 2H_2O$. When the nitrogen figure is calculated on the water-free substance the value agrees with that calculated for anhydrous copper aspartate. Found, 7.35 per cent; calculated, 7.19. Total yield of aspartic acid, 4.72 per cent.

The filtrate from the copper aspartate was freed from copper and another crop of hydrochloride was obtained in the usual way; weight, 0.37 gm.; per cent N, 12.51; calculated for glycine hydrochloride, 12.55. No further work was carried out on this fraction. The fact that glycine was found in this fraction confirms the experience of other investigators (11, 23).

Serine—Application of the periodate method (24) led to a figure of 4.37 per cent serine in the isinglass. The dimedon derivative had the correct melting point of 189°. Deduction of the amount of formaldehyde derived from hydroxylysine makes the corrected value 4.13 per cent.

Threonine—The method of Shinn and Nicolet (25) was used for the determination of threonine. A value of 3.22 per cent was obtained. Recovery experiments performed on serine and threonine by the periodate procedure gave results similar to those published by the authors quoted.

Alanine—An attempt was made to determine alanine by deamination, oxidation of the resulting lactic acid with ceric sulfate, and estimation of the acetaldehyde produced by absorption in sodium bisulfite and iodometric titration. The procedure involving deamination of alanine and determination of the resulting lactic acid was introduced by Kendall and Friedemann (26). Although recoveries of lactic acid were satisfactory (97 to 100 per cent), recoveries for alanine averaged only about 75 per cent. Since the α,β-dihydroxybutyric acid derived from the deamination of threonine also yields acetaldehyde on oxidation with ceric sulfate, recovery experiments were also run on this amino acid but as with alanine the results were low (70 per cent). The level of alanine in isinglass based on the total amount

of acetaldehyde evolved as described above less the amount derived from the threonine led to a value of 10.3 per cent. However, a more nearly correct figure is probably obtained by applying corrections for the recovery of alanine and threonine. The value so derived is 14.7 per cent.

Methionine and Cystine—An analysis of sulfur distribution was carried out by the Kassell and Brand modification (27) of Baernstein's method (28) with the following results: methionine 2.78 per cent (by volatile iodide),

TABLE I

Amino Acids of Isinglass

Amino acid	Isinglass		Gelatin	Bibliographic reference No.
	per cent of protein	*per cent of total N*	*per cent*	
Glycine...	9.55 (+)	9.79	27.0	31
Alanine.....	14.7	12.7	8.7	11
Serine . .	4.13	3 03	2.7	24
Threonine.... ..	3.22	2.08	1.4	25
Valine.	2.29	1.50	2.46	29
Leucine	3.76	2.21	3 30	29
Isoleucine	2.99	1.76	1.71	29
Aspartic acid	4.72	2.73	3.4	11
Glutamic "	8.19	4.27	5.8	11
Arginine	9.33	16 5	8 9	32
Histidine.	1.09	1.62	0 9	11
Lysine	4.35	4 58	5.9	11
Hydroxylysine.	0.36	0.34	0.9	18
Cystine	0.1	0.06	<0.1	*
Methionine. .	2.78	1.43	1.0	33*
Phernylalanine.... .	1.83	0.85	2.6	34
Ty osine...	0.0	0.0	0.0	11
Tryptophane	0 0	0.0	0 0	11
Proline	17.8	11 88	17.5	35
Hydroxyproline .	4.68	2.75	14.65	36
Ammonia.	0.68	3.09	0.4	11
	96 55	83 17	109 32	

* Beveridge, J. M. R., unpublished data.

2.62 per cent (by homocysteine); cystine, 0.1 per cent; inorganic sulfur, trace. By summation a figure of 0.630 per cent sulfur in isinglass is obtained. The value for total sulfur by the sodium peroxide fusion method was 0.632 per cent.

Valine, Leucine, Isoleucine—These were estimated by the microbiological method published recently by Kuiken, Norman, Lyman, Hale, and Blotter (29). The tomato eluate, which may be omitted under certain circum-

stances, was included in the test medium. The blank tube in the isoleucine series gave a fairly high titration value, indicating the probability that one (or more) of the amino acids used in making up the medium was contaminated with this material. In this connection it is interesting to note that Hegsted and Wardwell (30) have just reported that certain samples of synthetic *dl*-leucine actually contain some isoleucine. It was noted that at times there was a large variation in the results when the total volume of medium was titrated. However, if 5 ml. aliquots were taken as advised by Kuiken *et al.*, the titration values of duplicate tubes checked amazingly well, usually to within 0.05 ml. of 0.1 N sodium hydroxide. Apparently the occasional variation encountered when the total volume was used for the titration resulted from loss of medium through the wetting of plugs. The average values of a number of closely agreeing determinations follow: isoleucine 2.99 per cent, leucine 3.76 per cent, valine 2.29 per cent.

The results are summarized in Table I, in which the corresponding figures for gelatin are tabulated.

An examination of the data indicates that, with the exception of tryptophane, all of the essential amino acids are present. However, it is extremely doubtful whether all of them are present in sufficient amount to permit maximal growth (as determined by experiments on rats) even in the presence of an adequate amount of tryptophane. The values found for the essential amino acids parallel quite closely those quoted for gelatin, a protein which is considered to be deficient in valine, isoleucine, threonine, and methionine, besides tryptophane. It is noteworthy that isinglass contains markedly more methionine and threonine and may actually provide enough of these substances for maximal growth. It would therefore appear on the basis of the analytical data presented that isinglass is not so deficient as is gelatin in essential amino acids.

SUMMARY

1. Values for twenty amino acids in isinglass have been determined.

2. The totals of these values account for 96.6 per cent of the protein, or 83.2 per cent of the total nitrogen.

BIBLIOGRAPHY

1. Taylor, N. B., and Waters, E. T., *Canad. Med. Assn. J.*, **44**, 547 (1941).
2. Taylor, N. B., and Moorhouse, M. S., *Canad. Med. Assn. J.*, **49**, 251 (1943).
3. Hughes, F. N., Thesis, University of Toronto (1944).
4. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
5. Hopkins, F. G., and Cole, S. W., *Proc. Roy. Soc. London*, **68**, 21 (1901).

6. Block, R. J., and Bolling, D., The determination of the amino acids, Minneapolis, revised edition (1940).
7. Kapeller-Adler, R., *Biochem. Z.*, **252,** 185 (1932).
8. Vickery, H. B., *J. Biol. Chem.*, **132,** 325 (1940).
9. Bergmann, M., *J. Biol. Chem.*, **110,** 471 (1935).
10. Kapfhammer, J., and Eck, R., *Z. physiol. Chem.*, **170,** 294 (1927).
11. Dakin, H. D., *J. Biol. Chem.*, **44,** 499 (1920).
12. Van Slyke, D. D., Hiller, A., and Dillon, R. T., *J. Biol Chem.*, **146,** 137 (1942).
13. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem*, **79,** 377 (1928).
14. Kapeller-Adler, R., *Biochem. Z.*, **264,** 131 (1933).
15. Conrad, R. M , and Berg, C. P., *J. Biol. Chem.*, **117,** 351 (1937).
16. Woolley, D. W., and Peterson, W. H., *J. Biol. Chem.*, **122,** 207 (1937).
17. Vorländer, D., *Z. anal. Chem* , **77,** 241 (1929).
18. Van Slyke, D. D., Hiller, A., and MacFadyen, D. A., *J. Biol Chem.*, **141,** 681 (1941).
19. Schryver, S. B., Buston, H. W., and Mukherjee, D. H., *Proc. Roy. Soc. London,* *Series B,* **98,** 58 (1925).
20. Weber, C. J., *J. Biol. Chem.*, **86,** 217 (1930).
21. Van Slyke, D. D., Hiller, A., Dillon, R. T , and MacFadyen, D , *Proc. Soc. Exp Biol. and Med.*, **38,** 548 (1938).
22. Foreman, F. W., *Biochem. J.*, **8,** 463 (1914).
23. Jones, D. B , and Moeller, O., *J. Biol. Chem.*, **79,** 429 (1928).
24. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139,** 687 (1941).
25. Shinn, L A , and Nicolet, B. H., *J. Biol. Chem.*, **138,** 91 (1941).
26. Kendall, A I., and Friedemann, T. E., *J. Infect. Dis* , **47,** 171 (1930).
27. Kassell, B , and Brand, E., *J. Biol. Chem.*, **125,** 145 (1938)
28. Baernstein, H. D., *J. Biol. Chem.*, **115,** 33 (1936).
29. Kuiken, K. A., Norman, W. H., Lyman, C. M , Hale, F., and Blotter, L., *J. Biol. Chem* , **151,** 615 (1944).
30 Hegsted, D. M., and Wardwell, E. D., *J. Biol. Chem.*, **153,** 167 (1944).
31. Bergmann, M., and Stein, W H., *J. Biol. Chem* , **128,** 217 (1939).
32. Hunter, A., and Dauphinee, J. A., *J. Biol. Chem* , **85,** 627 (1930).
33. Baernstein, H. D , *J. Biol. Chem.*, **97,** 663 (1932).
34. Block, R. J , Jervis, G A., Bolling, D., and Webb, M., *J. Biol. Chem.*, **134,** 567 (1940).
35. Stein, W. H., and Bergmann, M., *J. Biol. Chem* , **134,** 627 (1940).
36. McFarlane, W. D., and Guest, G. H , *Canad. J Res., Sect B,* **17,** 139 (1939).

THE FUNCTION OF PYRIDOXINE AND PYRIDOXINE DERIVATIVES IN THE DECARBOXYLATION OF TYROSINE

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In a study of the tyrosine decarboxylation system of certain lactic acid bacteria, first described by Gale (1), Bellamy and Gunsalus (2) have reported that more pyridoxine and nicotinic acid are required for decarboxylase production than are needed for maximum growth. A medium of largely known composition has been obtained for the production of cells with strong decarboxylase activity (3) and a preliminary note on the function of the pyridoxine component in this system published (4).

Snell *et al.* (5) reported the presence of a compound in animal tissue which replaces pyridoxine in the nutrition of certain lactic acid bacteria. This substance, termed pseudopyridoxine, was also found in yeast extract which had been autoclaved with dilute hydrochloric acid. Material with similar activity can be formed from synthetic pyridoxine by autoclaving with amino acids, especially cystine (6), or by treatment with dilute hydrogen peroxide (7). While it is not certain that the substance produced by these three treatments is the same, the evidence favors this view. Therefore, until further evidence is available and to avoid the coining of new terms should the substances prove to be the same, pseudopyridoxine is used in the present paper to refer to the substance present in the preparations of Snell (6) and of Carpenter, Elvehjem, and Strong (7) which we have found to stimulate tyrosine decarboxylation by resting cells. This involves the assumption that the same active substance functions in the decarboxylation of tyrosine and in growth stimulation of *Streptococcus lactis* R and *Lactobacillus casei*[1] (No. 8043 and No. 7469, respectively, of the American Type Culture Collection).

In the present work the tyrosine decarboxylation system has been studied manometrically with phthalate buffer at pH 5.0 and resting cell suspensions as described in previous reports. By growing cells with suboptimum amounts of pyridoxine and adding substances to cell suspensions in Warburg cups, pseudopyridoxine has been shown to function in the tyrosine decarboxylase system.

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[1] Since this paper was submitted pyridoxal has been reported (Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944)) and shown to function in tyrosine decarboxylation (Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944)).

Methods

Cultures—Streptococcus faecalis, Strain 10C1, a typical enterococcus previously studied in some detail in this laboratory, was used in the original experiments. When it appeared that pseudopyridoxine was involved in the decarboxylation and difficulty was encountered in the preparation of active cells deficient only in this factor, another enterococcus, *Streptococcus lactis* R (No. 8043), was used. This culture has also been identified as a strain of *Streptococcus faecalis.*[2] Since Snell and Guirard (8) have shown that this culture will grow in the absence of pyridoxine, provided sufficient alanine is present, it is possible to obtain cells with a low pyridoxine, or pseudopyridoxine, content for a study of the decarboxylation system.

Media—In most of the experiments reported here the medium employed by Bellamy and Gunsalus (2) for the production of active cells of Strain 10C1 was used. With Strain R the pyridoxine was omitted, since gelatin in 1 per cent concentration provided about 10 mg. of alanine per tube, which is sufficient to replace pyridoxine (8). Cells of Strain R grown in this medium showed very slight tyrosine decarboxylase activity but were markedly stimulated by the addition of pseudopyridoxine to cell suspensions in the Warburg cups.

Manometric Experiments—Conventional Warburg methods were employed to determine the tyrosine decarboxylase activity. The cells were collected by centrifugation, resuspended in saline, and added to the side arms of the Warburg cups. As the $Q_{CO_2}(N)$ of these cells, with tyrosine, varies from 400 to 2000, depending upon the strain employed and the conditions of culture, from 10 to 2 mg. of cells were used per cup. In the main compartment the cups contained 0.5 ml. of M/30 suspension of tyrosine, 1 ml. of 0.075 M phthalate buffer, pH 5.0, and other factors, or water, to make 2.5 ml. The cells, in the side arms, were suspended in 0.5 ml. of saline. The gas phase was either air or 5 per cent $CO_2:N_2$. The buffer is sufficiently acid for the carbon dioxide to be released as formed. The rate of glycolysis was determined in M/30 bicarbonate buffer with 5 per cent $CO_2:N_2$ and 10 micromoles of glucose.

Results

To determine whether the metabolism of the cells in general is influenced by low growth levels of the pyridoxine, the glycolytic rate of cells harvested from the media with increasing levels of pyridoxine was compared with the rate of tyrosine decarboxylation. Glycolysis was selected as an energy-yielding mechanism intimately linked with the growth of the organism, whereas tyrosine decarboxylation appears to be a rather specific function whose importance to the organism is not as yet clear.

[2] Sherman, J. M., Niven, C. F., Jr., and Gunsalus, I. C , unpublished data.

As may be seen from Table I, the rate of glycolysis is about the same for cells harvested from media containing from 0.25 to 100 γ of pyridoxine per 10 ml. The lower level supported approximately maximum growth, in the medium used, when autoclaved 25 minutes at 15 pounds. On the other hand, the tyrosine decarboxylase activity of the cells increased with the level of pyridoxine to a maximum with about 50 γ per 10 ml. of medium. The large amounts required recall the results of Snell *et al.* (5) in which considerable quantities of pyridoxine were required for maximum growth, whereas only a small fraction of the pyridoxine present was removed during growth.

In order to determine whether pseudopyridoxine is the factor operating in the tyrosine decarboxylation system, 10 γ of pyridoxine were autoclaved

TABLE I

Influence of Pyridoxine Level in Growth Medium on Glycolysis and Tyrosine Decarboxylation by Streptococcus faecalis 10C1

In medium		Activity of cells	
Pyridoxine	Growth*	Glycolysis	Tyrosine decarboxylation
γ *per 10 ml.*		Q_G (N)	Q_{CO_2}(N)
0	4		
0.25	37	1400	60
0 5	37	1750	120
1 0	42	1750	290
5.0	44	1600	460
10.0	44	1550	870
20.0	43	1550	1300
50.0	44	1750	1600
100.0	44	1750	1300

* Nephelometer reading, each scale unit $\simeq$ 6 γ of bacterial N per 10 ml.

in the base medium, and the decarboxylase activity of the cells compared to that of cells harvested from media to which pyridoxine autoclaved alone, and with cystine, was added aseptically after autoclaving. The results, Fig. 1, show that the rate of decarboxylation was least with the pyridoxine added after sterilization, and greatest with the cystine-treated pyridoxine. With the pyridoxine autoclaved in the medium the cells showed intermediate activity. The decarboxylation rate also was maintained for a longer period with the cells from the medium containing cystine-treated pyridoxine. These results definitely point toward pseudopyridoxine as the active substance.

To determine whether pyridoxine and pseudopyridoxine would stimulate tyrosine decarboxylation by resting cells, cultures were grown with a sub-

optimum amount of pyridoxine (5 γ per 10 ml.), and the influence of these substances on the rate of decarboxylation determined (Fig. 2). The addition of 50 γ of cystine-treated pyridoxine to the Warburg flasks stimulated the decarboxylation rate more than 100 γ of the unheated vitamin. The original rate of decarboxylation was not greatly increased but the rate was maintained when pyridoxine or pseudopyridoxine was added. This may possibly indicate that the pyridoxine component functions as a prosthetic
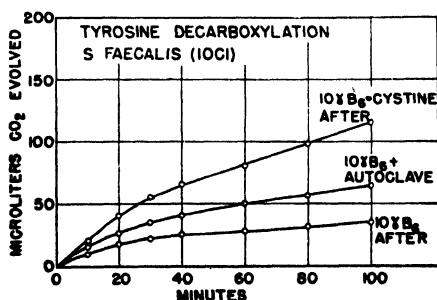
FIG. 1. Tyrosine decarboxylase activity of cells harvested from media containing pyridoxine autoclaved alone and added aseptically, with cystine in acetate buffer, and in the medium. The media were autoclaved 25 minutes at 15 pounds pressure.
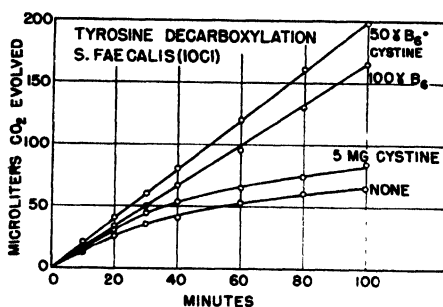
FIG. 2. Influence of pyridoxine and pseudopyridoxine on tyrosine decarboxylase activity of cell suspensions Per Warburg cup, 0.25 mg. of bacterial nitrogen, *Streptococcus faecalis*, Strain 10C1, grown with 5 γ of pyridoxine per 10 ml of medium.

group of the enzyme system and that higher concentrations are required to saturate the enzyme. In subsequent experiments it was found that unheated pyridoxine upon standing in the refrigerator slowly increased in activity. Therefore, it is possible that the stimulation shown by 100 γ of pyridoxine (Fig. 2) may have been due to traces of pseudopyridoxine in the preparation. Woolley[3] has also observed that, upon standing in the refrigerator, preparations containing pyridoxine increased in their growth-

[3] Woolley, D. W., personal communication (1944).

promoting activity for another enterococcus, *Streptococcus zymogenes* (No. H69D5).

With Strain 10C1 used in these experiments there is considerable decarboxylase in cells harvested from media containing 5 γ of pyridoxine per 10 ml. of medium. With lower amounts of pyridoxine in the growth medium the cells did not show stimulation in the Warburg cups. For this culture there is apparently still another factor needed for the production of active cells. As we have not been able thus far consistently to obtain cells deficient in pseudopyridoxine, which could be stimulated by the addition of this substance, another culture was sought as an alternative. Therefore, the *Streptococcus lactis* R was tested. When this culture is grown in a medium lacking pyridoxine, the cells show very little decarboxylase activity (Fig. 3). However, the addition of 20 γ of cystine-treated pyridoxine re-
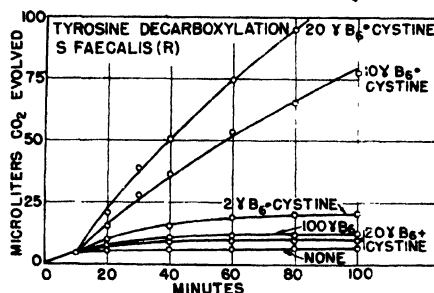
FIG 3 Influence of pyridoxine and pseudopyridoxine on tyrosine decarboxylation by cell suspensions of *Streptococcus faecalis*, Strain R   0 2 mg. of bacterial nitrogen per cup. The pyridoxine and cystine were autoclaved together 1 hour in acetate buffer; see the text.

sulted in the maximum rate of decarboxylation. (500 γ of pyridoxine were autoclaved for 1 hour at 15 pounds pressure with 10 mg. of cystine in 25 ml. of acetate buffer of pH 7.0 (7).) 10 γ of the cystine-treated pyridoxine gave more than half maximum stimulation and 2 γ showed some activity. With the two higher concentrations the decarboxylation rate was maintained over a period of 2 hours. As may also be seen from Fig. 3, no stimulation occurred with 100 γ of freshly prepared, unheated pyridoxine, nor with 20 γ of unheated pyridoxine plus 2 mg. of cystine. Freshly prepared solutions of some samples of synthetic pyridoxine show slight activity.

If pseudopyridoxine is the factor which functions in the tyrosine system of these cultures, samples prepared by the autoclaving of yeast extract in acid medium (5, 7) and by treatment with hydrogen peroxide (7) should also activate the decarboxylation. As shown in Fig. 4, both of these substances are active. The curve for 10 γ of cystine-treated pyridoxine from

Fig. 3 is included here for comparison. The figures in parentheses over the curves are the approximate pseudopyridoxine activity in the preparations as indicated by the results of Carpenter, Elvehjem, and Strong (7). While these figures are subject to some uncertainty, the stimulation by the different preparations corresponds reasonably well with the pseudopyridoxine content which these workers reported with *Lactobacillus casei* as the indicator organism. Yeast extract autoclaved at neutral reaction was not active in the stimulation of decarboxylation.

The maximum rate of decarboxylation obtained with Strain R (Q_{CO_2}(N) 400) was not so great as with Strain 10C1 (Q_{CO_2}(N) 1000) but the difference between the control rate, cells harvested from pyridoxine-free media, and that observed with the addition of pseudopyridoxine was much wider. With the lower control value stimulation occurred after a 10 minute lag
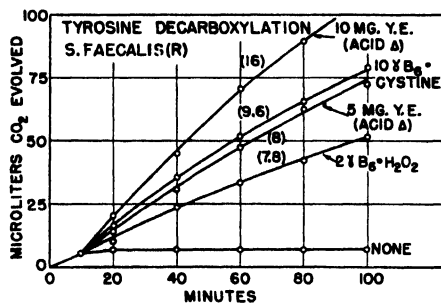
FIG 4 Influence of pseudopyridoxine preparations on tyrosine decarboxylation by cell suspensions of *Streptococcus faecalis*, Strain R   The 30 per cent yeast extract was autoclaved 1 hour in 0 1 N HCl; for the hydrogen peroxide-treated sample 5 γ per ml of pyridoxine were treated 4 hours at room temperature with 0 6 per cent H₂O₂; see (7).

and the rate was maintained for at least 2 hours. As pseudopyridoxine has not been isolated, it is not possible to judge the quantity required by the decarboxylation system. However, if as suggested by the results of Carpenter, Elvehjem, and Strong (7) not over 10 per cent of the pyridoxine is transformed to the active derivative by peroxide treatment, the quantity for half maximum stimulation in the decarboxylation rate is not over 0.2 γ per 3 ml. and is in all probability below this value.

After the various preparations had been tested for pseudopyridoxine content, they were stored in the refrigerator for future use. Over a period of 2 weeks the peroxide-treated pyridoxine continued to increase in activity and unheated pyridoxine became slightly active in the stimulation of decarboxylation. Pyridoxine upon aeration at room temperature for 18 hours (pH about 4.5) stimulated the decarboxylation. After 36 hours aeration the stimulation was double that found at 18 hours.

DISCUSSION

From the evidence available, the stimulation of tyrosine decarboxylation with cell suspensions of two strains of *Streptococcus faecalis* has been attributed to the pseudopyridoxine present in the preparations. Proof that the active material is the same in all the preparations is not available but present evidence favors this view. The action of this substance in an enzyme system would explain at least one of its biological functions. So far as we are aware this is the first known function of pyridoxine, or of its derivatives. It is not possible at present to state the value of this system from the standpoint of comparative biochemistry, since the mechanism of amino acid decarboxylation, especially tyrosine, in other cells is not known.

It seems possible that this type of system may be of importance in animal tissue in the metabolism of tyrosine which has been associated with hypertension (9). This view is strengthened by the observation of Bernheim and Bernheim (10) that tyramine, but not tyrosine, is deaminated by most rat tissues.

The numerous factors involved in the formation and function of the system require further study. Among the factors of importance for the formation of the system are the presence of tyrosine, the final pH of growth, the concentration of nicotinic acid, and apparently an unidentified factor present in tryptone and yeast extract, in addition to the pyridoxine component.

SUMMARY

A derivative of pyridoxine present in acid-autoclaved yeast extract and in pyridoxine solutions treated with cystine, or with dilute hydrogen peroxide, has been shown to function in the decarboxylation of tyrosine. The stimulation of the tyrosine decarboxylase system by these three preparations is in proportion to their pseudopyridoxine content as reported by Carpenter, Elvehjem, and Strong.

Unaltered pyridoxine is inactive or much less active in this function.

Pyridoxine upon standing in the refrigerator or upon aeration gradually increases in ability to stimulate the decarboxylase system.

BIBLIOGRAPHY

1. Gale, E. F., *Biochem. J.*, **34**, 846 (1940).
2. Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **46**, 573 (1943).
3. Bellamy, W. D., and Gunsalus, I. C., *J Bact.*, in press (1944).
4. Gunsalus, I. C , and Bellamy, W. D., *J. Bact.*, **47**, 413 (1944).
5. Snell, E. E., Guirard, B. M., and Williams, R J., *J. Biol. Chem.*, **143**, 519 (1942).
6. Snell, E. E , *Proc. Soc. Exp. Biol and Med.*, **51**, 356 (1942).
7. Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc Exp. Biol. and Med*, **54**, 123 (1943).
8 Snell, E. E , and Guirard, B. M., *Proc. Nat. Acad Sc*, **29**, 66 (1943).
9. Martin, G J., Abstracts, American Chemical Society, 107th meeting, Cleveland, 10B (1944).
10. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **153**, 369 (1944).

THE VERATRINE ALKALOIDS

XXII. ON PSEUDOJERVINE AND VERATROSINE, A COMPANION GLYCOSIDE IN VERATRUM VIRIDE

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In the course of their studies on the alkaloids contained in *Veratrum album* and *Veratrum viride*, Wright and Luff (1) isolated a new, sparingly soluble base called pseudojervine because of the similarity to jervine in the color developed by its solution in sulfuric acid. The formulation, $C_{29}H_{43}O_7N$, which was derived from their analytical data has more recently been altered by Poethke (2) to $C_{33}H_{49}O_8N$ on the basis of titration results and analytical data obtained with a number of its derivatives. Poethke also demonstrated the secondary character of its nitrogen atom by the preparation of a nitroso derivative. Because of the resemblance to jervine in this respect and because of its similar behavior with sulfuric acid, he accepted the view of Wright and Luff of a close relationship between the two alkaloids. However, no clue to the nature of this relationship was given. In a previous paper (3), in which the formulation of jervine was revised to $C_{27}H_{39}O_3N$, we have discussed the possibility that pseudojervine, with the formula $C_{33}H_{49}O_8N$, could be a glycoside of the simpler base with a hexose. We have since had opportunity to verify this relationship by the study of pseudojervine itself.

Following a number of unsuccessful attempts to isolate this alkaloid from the sample of commercial *Veratrum album* with which we had been working, we turned to an investigation of a commercial sample of *Veratrum viride* of American origin. This study has resulted in the isolation of a number of new alkaloids from this source, and the need for a revision of certain previously recorded interpretations. The crude plant material was at first well extracted with benzene essentially in the manner previously described by us for the extraction of *Veratrum album* (4). The results of the study of the large alkaloid fraction contained in this extract will be described in a subsequent communication. Following the benzene extraction, the crude material was then further extracted with alcohol. When this extract was investigated, an additional crude mixture of bases was obtained from which pseudojervine was readily isolated. It was found to agree in general properties with those previously recorded for the substance.

Its behavior toward acid demonstrated its glycosidic character. How-

ever, the base which was isolated from the hydrolysis mixture did not yield the sparingly soluble sulfate or hydrochloride characteristic of jervine. When the free base was extracted with chloroform, it was obtained in a form which contained chloroform of crystallization. It crystallized from acetone also with solvent of crystallization. Under proper conditions, it separated from diluted ethanol, likewise with solvent. Attempts to obtain the base by direct crystallization in a solvent-free form have not been successful. In each case, the solvent-containing base possessed a low and unsatisfactory softening point, rather than a melting point. From acetone, it crystallized with 1 mole of solvent and gave the rotation $[\alpha]_D^{30} = -32.5°$ in ethanol. The analytical data with the solvent-free base showed it to be an isomer of jervine, $C_{27}H_{39}O_3N$. In sulfuric acid, its behavior was indistinguishable from that of jervine as regards the character and succession of colors produced. For convenience, the trivial name, *isojervine*, has been adopted. The same base was obtained by cleavage of pseudo-jervine at room temperature with methanol saturated with HCl.

The failure to obtain jervine itself suggested at once the possibility that jervine may be isomerized to the iso compound under the conditions used for the cleavage of pseudojervine. This was found to be the case. Jervine yielded a substance indistinguishable in properties by this treatment. It appears that either isojervine exists as such in pseudojervine, or, what is more probable, that it is an artifact and pseudojervine is a derivative of jervine itself.

A product also of the hydrolysis of pseudojervine was found to be *d*-glucose. The latter was isolated as the phenylosazone, which was characterized as the glucose derivative by its mutarotation in pyridine-alcohol solution, according to Levene and La Forge (5). The specific rotation, $[\alpha]_D = +47°$, found for the sugar solution itself approximated that for *d*-glucose; *viz.*, $[\alpha]_D = +52.5°$.

Investigation of the mother liquors from pseudojervine has led to the isolation of a new glycosidic alkaloid which proved to be somewhat more soluble in alcohol than pseudojervine. The formulation derived from the analytical results was $C_{33}H_{49}O_7N$, or with 1 oxygen atom less than the number contained in pseudojervine. The substance formed woolly masses of needles from dilute alcohol which contained solvent. The rotation found was $[\alpha]_D^{25} = -53°$ ($c = 0.255$ in chloroform-ethanol). On hydrolysis with dilute HCl, it yielded the sparingly soluble hydrochloride of a base. Analysis of the base itself gave figures which approximated the formulation, $C_{27}H_{39}O_2N$, which differs from that of jervine by 1 oxygen atom. The sugar formed was identified again as *d*-glucose by the specific rotation of its aqueous solution; *viz.*, $[\alpha]_D = +54°$. This was substantiated by the analysis and mutarotation of its phenylosazone.

The specific rotation of the base, $C_{27}H_{39}O_2N$ in methanol, was found to be $[\alpha]_D^{33} = -69°$. The occurrence of the new glycoside with pseudojervine recalled the recent isolation by Saito (6) from *Veratrum grandiflorum* Loes. fil. of the new companion alkaloid to jervine, veratramine, for which the formulation $C_{26}H_{35}O_2N$ was derived. In a study to be presented in another connection, veratramine was also isolated from *Veratrum viride*. As will be shown, the analytical data obtained with this alkaloid and its derivatives have made necessary a revision of its formulation to $C_{27}H_{39}O_2N$. The base obtained by the hydrolysis of the above glycoside was found to bear a close resemblance in properties to veratramine. In particular, the substance recovered after veratramine itself was subjected to the action of HCl was indistinguishable in properties from the new base. The conclusion appears, therefore, justified that the new glycoside is a derivative of veratramine. The trivial name, *veratrosine*, has been adopted for it.

The occurrence of the two glycosidic alkaloids, pseudojervine and veratrosine, which are derivatives of steroidal bases, brings to mind the closely related glycosidic potato alkaloids, such as solanine.

EXPERIMENTAL

A commercial sample of the roots and rhizomes of *Veratrum viride* was obtained from S. B. Penick and Company. The ground crude mass, in amounts of 2 kilos, was moistened with dilute ammonia and first extracted with benzene, essentially as previously described by us (4). The results of the study of this extract will be presented at a later time. The remaining material was at once covered with 6 liters of 95 per cent ethanol and, after standing at room temperature for a day or so, was filtered and pressed off. The solid was again suspended in 4 liters of solvent to which 50 cc. of ammonia (0.9 sp. gr.) had been added. The extraction was repeated a third time with 4 liters of alcohol. The combined extracts were concentrated *in vacuo* to remove all alcohol. Toward the end, the addition of small amounts of octyl alcohol was found necessary to control the foaming. The very dark colored, resinous, aqueous mixture was treated with an excess of 20 per cent sodium carbonate solution, and shaken with an equal volume of chloroform. For separation, the mixture was allowed to stand overnight. The rather large fraction of undissolved, resinous material was macerated with about 250 cc. of water, then added to the original aqueous phase, and extracted with fresh chloroform. Again a resin formed at the interface, and its extraction with chloroform, after suspension in water, was repeated several times. In this manner, from a total of 6 kilos of root, about 5 liters of combined chloroform extract resulted. After this mixture was washed with water, it was repeatedly extracted with 2.5 per cent tartaric acid solution. A resinous suspension, which collected at

the interface, was separated by centrifugation and reextracted with acid after resuspension. The combined, clear, colored, acid extract was made alkaline with excess NaOH and reextracted with chloroform. The latter was cleared with sodium sulfate and then concentrated to a thin syrup. Addition of ether gave a powder of crude, mixed, alkaloidal material. The yield, which varied considerably because of manipulative difficulties, approximated 20 gm. from 6 kilos of original plant material.

Pseudojervine[1]—15 gm. of the crude alkaloid mixture were warmed with 75 cc. of 95 per cent ethanol. Solution was followed by rapid crystallization of a fine, colored powder. The yield of what proved to be pseudojervine was 3.5 gm. For recrystallization, it was dissolved in a mixture of 50 cc. of methanol and 3 cc. of acetic acid. After being cleared with bone-black, the solution was treated with an excess of ammonia and then carefully diluted for crystallization. A repetition of the process yielded lustrous leaflets, which melted with decomposition at 300–301° after preliminary softening and discoloration.

$C_{33}H_{49}O_8N$ Calculated, C 67.42, H 8 41; found, C 67.59, H 8.35

The rotation was determined in a mixture of 1 volume of absolute ethanol and 3 volumes of chloroform. $[\alpha]_D^{25} = -133°$ ($c = 0.46$).

Poethke (2) reported for a different ethanol-chloroform (7:43) mixture $[\alpha]_D^{20} = -139°$ ($c = 0.4$).

Seiferle, Johns, and Richardson (7) reported $[\alpha]_D^{23} = -133.4°$ ($c = 0.48$ in 1:3 ethanol-chloroform).

Cleavage of Pseudojervine. Isojervine—0.2 gm. of the glycoside was refluxed for 1 hour and 20 minutes in 20 cc. of 2 per cent aqueous HCl. The solution remained clear on cooling. It was made just alkaline to phenolphthalein and repeatedly extracted with a fair volume of chloroform. The latter, after being cleared with sodium sulfate, was concentrated to a few cc. Crystallization of the sparingly soluble chloroform compound of isojervine occurred. The yield was 0.13 gm. It formed glistening micro leaflets or platelets which tenaciously retained color. It sintered above 100° and gradually softened from 135–150°, depending on the rate of heating. Analysis in this form was rendered difficult by the retention of solvent.

The desiccator-dried material gave the following figures on direct analysis.

$C_{27}H_{39}O_3N \cdot CHCl_3$.   Calculated, C 61 69, H 7 40, found, C 60 45, H 7.33

Better results were obtained with the substance which crystallized from about 50 per cent alcohol. It formed microscopic, short, prismatic needles,

[1] We wish to thank Dr I. B. Johns, of the Iowa State College, for a small sample of pseudojervine.

which also contained solvent. It gradually softened above 108°, and slowly melted to a resin at 114–116° with effervescence. This form appeared to contain mostly alcohol of crystallization.

$C_{27}H_{39}O_3N \cdot C_2H_5OH$. Calculated, OC_2H_5 9.58; found, 8.57

The substance, when dried at 90° and then at 110° and 2 mm., lost 9.12 per cent of solvent.

$C_{27}H_{39}O_3N$. Calculated, C 76.18, H 9.24; found, C 75.98, H 9.27

The most satisfactory form of the base for characterization was obtained from dry acetone. The data recorded here came from the material which resulted from the cleavage of pseudojervine with methanol saturated at 0° with HCl. For this purpose, 0.75 gm. of pseudojervine was dissolved in 10 cc. of this mixture and left at room temperature for 1 hour. The clear solution was concentrated and the residue, after treatment with water and an excess of sodium carbonate solution, was at once extracted with a large volume of chloroform. Occasionally, the chloroform compound crystallized during this operation. The substance, which agreed in all properties with that described above, was dissolved in warm alcohol and concentrated to a resin. The latter was dissolved in dry acetone and yielded sparingly soluble flat prisms or plates which contained solvent. It melted at 110–114° with effervescence after preliminary sintering. $[\alpha]_D^{30} = -32°$ ($c = 1.11$ in 95 per cent ethanol). For analysis, it was dried first at 90°, followed by 110° at 2 mm.

$C_{27}H_{39}O_3N \cdot C_3H_6O$. Calculated, C_3H_6O 12.00, found, 11.63

$C_{27}H_{39}O_3N$. Calculated, C 76 18, H 9.24, found, C 76.07, H 9.23

Contrary to jervine, this substance does not yield a sparingly soluble sulfate or hydrochloride.

When 0.5 gm. of jervine was substituted for pseudojervine with methyl alcoholic HCl, the yield of chloroform compound was 0.46 gm. The substance separated from acetone as platelets or prisms indistinguishable from that described above. It gradually melted with effervescence at 110–113°, after preliminary sintering. $[\alpha]_D^{30} = -32°$ ($c = 1.01$ in 95 per cent ethanol).

The desiccator-dried substance gave the following figures.

$C_{27}H_{39}O_3N \cdot C_3H_6O$. Calculated, C 74 48, H 9.38, found, C 74.20, H 9.25

When dried at 90° and then at 110° and 2 mm., it lost its acetone.

$C_{27}H_{39}O_3N \cdot C_3H_6O$. Calculated, C_3H_6O 12.00, found, 11.95

$C_{27}H_{39}O_3N$. Calculated, C 76.18, H 9.24; found, C 76.31, H 8.99

Because of the sparing solubility of its hydrochloride in aqueous HCl, 0.5 gm. of jervine was boiled instead in 50 per cent ethanol, which contained

2 per cent HCl. Practically all dissolved at once. When refluxed for 1.5 hours and allowed to cool, crystallization again occurred. 0.35 gm. of jervine hydrochloride was recovered and identified by reconversion to the base. The soluble hydrochloride fraction was extracted as the free base with chloroform. 0.17 gm. of the chloroform compound of isojervine was obtained. After recrystallization from acetone, it agreed in all properties with the base obtained from pseudojervine. The substance as directly obtained from acetone was analyzed.

<div align="center">Found, C 74.14, H 9.45</div>

d-Glucose—In the case of the hydrolysis of pseudojervine with aqueous acid, as given above, the alkaline aqueous phase, which remained after extraction with chloroform, was at once exactly neutralized to litmus with HCl and, after being cleared with norit, was concentrated *in vacuo* to dryness. The residue was extracted with 95 per cent alcohol. The extract after concentration to dryness was dissolved in water and made up to volume. The sugar determination with copper solution showed the presence of 1.158 per cent calculated as glucose. The observed rotation was $[\alpha]_D^{30} = +0.54°$. From this, $[\alpha]_D^{30} = +47°$. For *d*-glucose, $[\alpha]_D = +52.5°$.

The phenylosazone substantiated this result. 71 mg. of the latter were obtained from the sugar fraction from 0.3 gm. of pseudojervine. After recrystallization, it melted with decomposition at 205–206°.

<div align="center">$C_{18}H_{22}O_4N_4$.   Calculated, C 60.30, H 6.19; found, C 60.29, H 6.43</div>

The mutarotation of the osazone in pyridine-alcohol solution was also determined according to Levene and La Forge (5), except that the final reading was taken after 44 hours instead of 24 hours   The initial reading was $[\alpha]_D^{28} = -66°$; after 44 hours, $[\alpha]_D^{28} = -30°$.

Veratrosine—The mother liquor from the above 3.5 gm. of crude pseudojervine was carefully diluted. A small amount of suspension of very fine crystals, apparently additional pseudojervine, appeared but, since its removal by filtration proved difficult, the dilution of the mixture was carried further to incipient turbidity. After several days, a somewhat colored, crystalline deposit was obtained, which amounted to 6.8 gm. This material was dissolved in 100 cc. of methanol with the aid of 4 cc. of 50 per cent acetic acid   The colored solution was treated with 5 cc. of ammonia and allowed to stand. Aside from flocculent material, no crystallization occurred. The mixture was filtered and washed with methanol. To the solution of 150 cc., 50 cc. of H_2O were added. Gradual crystallization then occurred. After standing for completion, the deposit was collected with methanol-water (3:1) in which it appeared to be somewhat soluble. 3.1

gm. of substance were obtained, which sintered above 210° and gradually softened to a melt at 225–231°. For further purification, this was dissolved in 25 cc. of hot methanol and filtered rapidly from a small amount of sparingly soluble material. The filtrate which began to crystallize was carefully diluted. Prompt separation of woolly masses of delicate needles occurred. About 1 gm. was obtained. After preliminary discoloration and sintering, especially above 230°, it softened above 235° and finally became a colored, effervescent mass at 242–243°. The melting point varied somewhat with individual preparations and also depended on the rate of heating and the conditions of crystallization.

Because of its sparing solubility in most solvents except methanol, the rotation of the alkaloid was taken in a mixture of equal volumes of 95 per cent ethanol and chloroform. $[\alpha]_D^{25} = -53°$ $(c = 0.255)$.

For analysis, the substance was dried at 110° and 2 mm. The dried material proved to be somewhat hygroscopic.

$C_{33}H_{49}O_7N$. Calculated. C 69.31, H 8 64
Found. (a) " 69 29, " 8 56
" (b) " 69 19, " 8.64
" (c) " 68 78, " 8 87

Cleavage of Veratrosine—0.3 gm. of the glycoside was refluxed in 60 cc. of 2 per cent aqueous HCl for 1 hour. The clear solution gradually deposited a crystalline salt on cooling. After 24 hours, the collected material amounted to 0.17 gm. The aqueous solution proved to contain a small amount of unhydrolyzed glycoside and the sugar fraction. The above hydrochloride was dissolved in a good volume of hot water, rapidly cooled, and at once made alkaline and extracted quickly with chloroform before coagulation. The dried extract yielded a residue of amorphous base which crystallized from dilute alcohol as delicate needles. The substance gradually melted at 192–195°, but did not clear until 199°. It showed no depression when mixed with veratramine. $[\alpha]_D^{33} = -69°$ $(c = 0.99$ in methanol).

For analysis, the base was dried at 115° and 2 mm. The hygroscopic character of the dried material rendered analysis rather difficult.

$C_{27}H_{39}O_2N$. Calculated, C 79.16, H 9.60; found, C 78.90, H 9 91

The aqueous filtrate from the above hydrochloride was carefully treated with alkali until phenolphthalein just turned. The flocculent precipitate was extracted with chloroform and the aqueous sugar solution was carefully neutralized with HCl. The chloroform extract yielded a small amount of unhydrolyzed glycoside which crystallized as delicate needles from the concentrated chloroform solution. After recrystallization from

methanol, it exhibited the usual melting point and properties, and gave the following figures.

<p align="center">Found, C 69.04, H 8.73</p>

The sugar solution was treated in the manner described in the case of pseudojervine, and brought to volume. The sugar content of the solution calculated as glucose was found to be 1.059 per cent. The observed rotation was $[\alpha]_D^{30} = +0.57°$. From this, $[\alpha]_D^{30} = +54°$. The phenylosazone melted at 205–207°.

<p align="center">$C_{18}H_{22}O_4N_4$. Calculated, C 60.30, H 6.19; found, C 60.22, H 6.18</p>

The initial rotation of the osazone in pyridine-alcohol was found to be $[\alpha]_D^{28} = -62°$; after 44 hours, $[\alpha]_D^{28} = -30°$. The sugar is, therefore, d-glucose.

The analytical results reported here were obtained by Mr. D. Rigakos.

BIBLIOGRAPHY

1. Wright, C. R. A , and Luff, A. P., *J. Chem. Soc.*, **35**, 405 (1879).
2. Poethke, W., *Arch. Pharm.*, **276**, 170 (1938).
3. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **148**, 51 (1943).
4. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **143**, 427 (1942).
5. Levene, P A., and La Forge, F. B., *J. Biol. Chem.*, **20**, 429 (1915).
6. Saito, K., *Bull. Chem. Soc. Japan*, **15**, 22 (1940).
7. Seiferle, E J., Johns, I B , and Richardson, C. H., *J. Econ. Entomol.*, **35**, 35 (1942).

THE PREPARATION OF FRACTIONS FROM PANCREAS THAT PREVENT FATTY LIVERS IN DEPANCREATIZED DOGS MAINTAINED WITH INSULIN

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Previous work has demonstrated that raw pancreas contains a factor that prevents fatty livers in completely depancreatized dogs maintained with insulin (1–3). Although choline in large amounts has a similar effect (4, 5), it was demonstrated by a procedure in which the lipotropic effects of choline and pancreas were compared that pancreas is still active in amounts that cannot be accounted for by the choline content (3).

In 1936 Dragstedt and his coworkers extracted pancreas with alcohol and obtained a fraction (lipocaic) which they claimed to be a new hormone capable of preventing and curing fatty livers in completely depancreatized dogs maintained with insulin (6). This fraction was reported to be soluble in 60 per cent alcohol. It was found by Entenman *et al.*, however, that lipocaic[1] is a poor source of the antifatty liver (A. F. L.) factor of the pancreas (7). An amount of lipocaic derived from 100 gm. of raw pancreas was shown to be less effective than 1 gm. of Fraction AR,[2] a dried defatted fraction derived from 5.5 gm. of raw pancreas.

The present report deals with procedures for the isolation of the A. F. L. factor of the pancreas. The fractions prepared here differ from that used by Dragstedt and his coworkers in that they were first extracted with dilute acid and then precipitated with ammonium sulfate. The daily ingestion of as small an amount as 60 mg. of one of the fractions contained sufficient activity to maintain normal livers in depancreatized dogs for as long as 6 months.

Fractionation Procedures

Fraction A—Fresh beef pancreas was freed of extraneous tissue and thoroughly ground in a small meat grinder. 1 kilo of this ground tissue

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[1] The term lipocaic is reserved here for Dragstedt's fraction of pancreas.

[2] Fraction AR was prepared as follows. Fresh pancreas was ground and thoroughly shaken with 2 volumes of acetone for 1 hour. The residue was extracted a second time with the same amount of acetone and then pressed to remove as much of the solvent as possible. The residue was then dried in warm air, ground to a fine powder, and finally extracted with ethyl ether in a Soxhlet apparatus for 8 hours. It was stored at −18° until used.

was then mixed with 2000 cc. of 0.24 N H_2SO_4 and the mixture agitated at room temperature for 30 minutes in a mechanical shaker. It was then allowed to stand overnight (18 hours) at 4°. At the end of this time the mixture was shaken for 30 minutes at room temperature and squeezed through coarse muslin. Approximately 2100 cc. of a reddish turbid liquid were obtained, and to it were added 377 gm. of solid c.p. ammonium sulfate. The ammonium sulfate added was sufficient to yield approximately 0.25 saturation. The mixture was shaken at room temperature until the ammonium sulfate dissolved and then placed in a cold room (4°) for 12 hours. The mixture was then filtered in the cold (4°) with suction through a large Büchner funnel containing No. 1 Whatman filter paper and a layer (1 gm. in all) of Super-Cel. The filtration required approximately 18 to 24 hours for completion. The residue was discarded.

377 gm. of solid ammonium sulfate were then added to the filtrate and the mixture shaken until the salt completely dissolved. This raised the concentration of ammonium sulfate to approximately 0.5 saturation. The white precipitate that formed was allowed to settle for 12 hours in the cold room. The mixture was then filtered with suction through a large Büchner funnel containing No. 1 Whatman filter paper and 1 gm. of Super-Cel. This filtration, which was carried out in the cold room, required about 1 hour. The filtrate was discarded. The precipitate was dried by suction; great care was taken to avoid excessive drying. This precipitate is designated here Fraction A. 1 kilo of raw pancreas yielded approximately 19 gm. of Fraction A.

For convenience of feeding, a dried preparation of Fraction A was made as follows: An amount of Fraction A derived from 1000 gm. of pancreas was suspended in 80 cc. of distilled water and 400 cc. of acetone added. The precipitate formed was allowed to settle for 4 hours and separated by filtration. This precipitate was dried in air for 2 hours at room temperature and then stored in a desiccator at 4° until fed. The material so obtained was a grayish white powder.

Fraction B—An amount of Fraction A derived from 1000 gm. of pancreas was dissolved in 350 cc. of distilled water and transferred to the cold room (4°). It was made 0.25 saturated with respect to ammonium sulfate; this was done by adding to it slowly and with stirring a solution that had been saturated with ammonium sulfate at 4°. The mixture was then kept in the cold room at 4° for 12 hours. It was filtered with suction through a No. 1 Whatman filter paper and 1 gm. of Super-Cel in a large Büchner funnel. The precipitate was discarded. To the filtrate was added more of the cold (4°) saturated solution of ammonium sulfate, so as to make the filtrate 0.5 saturated with respect to ammonium sulfate. The mixture was set aside in the cold room (4°) for 12 hours. It was then filtered as de-

scribed above. The filtrate was discarded and the precipitate dried by suction. Care was taken to avoid excessive drying of this residue. It was suspended in 80 cc. of distilled water and to it 400 cc. of acetone were added. The mixture was allowed to stand at room temperature for 4 hours. The white precipitate that settled out was separated by filtration as described above. The residue is Fraction B. It was thoroughly dried in air and stored in a desiccator at 4° until it was fed. 1 kilo of raw pancreas yielded approximately 15 gm. of Fraction B.

Fraction 27C—An amount of Fraction A derived from 1000 gm. of pancreas was suspended in about 200 cc. of water and dialyzed for 48 hours in running tap water in order to free it from ammonium sulfate. The mixture was then centrifuged. The small amount of residue (Fraction 27B) was found inactive. The supernatant (pH 5.9) was made up to 500 cc. with water and an equal volume of ·a cold saturated solution of ammonium sulfate added slowly with stirring. A white precipitate formed; it was allowed to settle in the cold room for 12 hours. The precipitate was separated by filtration and then treated with 1 volume of water and 5 volumes of acetone, as described for Fractions A and B. The product obtained was a white powder; 1000 gm. of pancreas yielded approximately 12 gm. of this fraction, which is designated here Fraction 27C.

Bioassay of Pancreatic Fractions

The method employed in this laboratory for the assay of these fractions by the use of completely depancreatized dogs has been described elsewhere (8). Weighed amounts of each fraction were fed twice daily with the diet for as long as 20 weeks and the whole liver excised at the end of this time for fat analysis. The ability of a fraction to prevent the deposition of abnormal amounts of fat in the liver for as long as 5 months (as shown by the finding of a normal fat content in the liver at the end of the period of assay) is taken as evidence for the presence of the A. F. L. factor in the fraction fed.

A positive result with this assay procedure means that the amount of a fraction fed contains a sufficient amount of A. F. L. factor to maintain a normal fat content in the liver for long periods. Amounts of the A. F. L. factor that are not sufficient for complete prevention of fatty livers will not be detected by this procedure. But no other procedure of assay in the depancreatized dog can be justified at present.

Results

In Table I are shown the effects of the daily feeding of 240 to 380 mg. of Fractions A, B, and 27C. The amounts fed were derived from 20 gm. of raw pancreas. These amounts were sufficient to prevent the appearance of

abnormal amounts of fat in the livers of depancreatized dogs for as long as 5 months. The activity of 240 mg. of Fraction 27C was tested in six dogs.

TABLE I

Effect of Feeding Various Fractions Derived from 20 Gm. of Raw Pancreas

Dog No.	Pancreas fraction*			Liver		Body weight	
	Fraction No.	Amount fed	Period fed	Weight	Total fatty acids	Preoperative	Final
		mg	*wks.*	*gm.*	*per cent*	*kg.*	*kg.*
D334	A	380	20	359	2.7	15.0	9.8
D335	A	380	13	459	2.7	14.8	10.2
D336	A	380	20	374	5.8	13.2	8.9
D327	B	300	20	453	4.4	8.0	6.2
D329	B	300	20	529	3.9	12.0	7.2
D378	27B	†	12	281	30.5	9.8	7.8
D379	27B	†	15	490	22.0	12.5	6.6
D381	27B	†	16	502	25.0	14.0	9.0
D370	27C	240	21	422	1.7	12.0	8.5
D390	27C	240	16	430	6.5	11.7	8.8
D394	27C	240	17	586	2.7	15.3	10.9
D410	27C	240	20	473	2.6	17.5	13.1
D414	27C	240	20	551	2.4	15.0	11.7
D429	27C	240	13	470	4.0	9.7	8.3

* These fractions were prepared fresh every 3 weeks. All fractions contained small amounts of Super-Cel.

† Prepared as a suspension that was kept frozen until used.

TABLE II

Effect of Feeding 60 Mg. of Fraction 27C Derived from 5 Gm. of Raw Pancreas

Dog No.	Period fraction fed*	Liver		Body weight	
		Weight	Total fatty acids	Preoperative	Final
	wks.	*gm.*	*per cent*	*kg*	*kg.*
D431	10	600	3.6	15.2	9.7
D434	12	372	3.1	16.2	10.0
D427	26	452	3.1	12.7	10.3
D428	26	460	3.1	11.7	8.0
D440	26	472	3.4	11.5	9.3

* A large amount of Fraction 27C was prepared at one time for this experiment. It was stored in the ice box until used.

In five of them the fatty acid content of the liver did not exceed 4 per cent; in one dog the fatty acid content found after 16 weeks of daily feeding was 6.5 per cent. Fraction 27B was found completely inactive; 30, 22, and 25

per cent fatty acids were present in the livers of three dogs that had been fed this fraction for 12, 15, and 16 weeks respectively.

The potency of Fraction 27C is shown in Table II. The daily feeding of as small an amount as 60 mg. of this fraction served to prevent fatty infiltration of the liver for over 6 months. In three dogs that received this amount of Fraction 27C for 6 months, the total fatty acid content of the liver did not exceed 3.4 per cent. 60 mg. of Fraction 27C were obtained from 5 gm. of pancreas; it was shown previously that the feeding of 5 gm. of raw pancreas prevents fatty livers in depancreatized dogs maintained with insulin.

In the small amounts fed, none of the fractions maintained the initial weight of the dogs (Tables I and II). It must be obvious, however, that factors in addition to (or perhaps other than) the A. F. L. factor are concerned with the nutritional state of a dog deprived of the external secretion of the pancreas. Only the A. F. L. activity of the pancreatic fractions fed is measured here by the method of assay used.

Comment

The A. F. L. factor of the pancreas is soluble in dilute acid. All active fractions used in this study were obtained from pancreas by treatment with 0.24 N H_2SO_4. The results obtained with Fractions A, B, and 27C leave no doubt that the active factor is insoluble in concentrations of ammonium sulfate between 0.25 and 0.5 saturation. In the preparation of Fraction A, the limits of the concentrations of ammonium sulfate were roughly fixed between 0.25 and 0.5 saturation by the addition of solid ammonium sulfate. In the preparation of Fractions B and 27C the limits were more carefully fixed by a second precipitation with saturated solutions of ammonium sulfate. Fraction B contained none of the material insoluble in 0.25 saturated ammonium sulfate, whereas Fraction 27C contained the material insoluble in concentrations of ammonium sulfate not greater than 0.5 saturation.

The presence of activity in Fraction 27C shows that the A. F. L. factor in pancreas is not dialyzable. The precipitate that settles out during the dialysis (Fraction 27B) apparently contains none of the active factor. This conclusion is based not only on the inability of Fraction 27B to prevent fatty livers when fed in amounts derived from 20 gm. of raw pancreas, but also on its failure to raise the blood lipids of depancreatized dogs maintained with insulin; the daily feeding of an amount of Fraction 27B derived from 200 gm. of pancreas for 3 weeks failed to raise the blood lipids of depancreatized dogs maintained with insulin. It has been shown elsewhere that the feeding of 1 gm. of raw pancreas per day is sufficient to raise the blood lipids of such animals (9).

SUMMARY

1. The preparation of pancreatic fractions that prevent fatty livers in completely depancreatized dogs maintained with insulin is described.

2. Active fractions were obtained from dilute acid extracts of pancreas by precipitation with ammonium sulfate between concentrations of 0.25 and 0.5 saturation.

3. The daily feeding of as small an amount as 60 mg. of a fraction maintained a normal fat content in the livers of completely depancreatized dogs for as long as 6 months.

BIBLIOGRAPHY

1. Allen, F. N., Bowie, J. J., Macleod, J. J. R., and Robinson, W. L., *Brit. J. Exp. Path.*, **5**, 75, (1924).
2. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **119**, 435 (1937).
3. Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **138**, 477 (1941).
4. Best, C. H., Ferguson, G. C., and Hershey, J. M., *J. Physiol.*, **79**, 94 (1933).
5. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **120**, 647 (1937).
6. Dragstedt, L. R., Van Prohaska, J., and Harms, H. P., *Am. J. Physiol.*, **117**, 175 (1936).
7. Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *Am. J. Physiol.*, **141**, 221 (1944).
8. Montgomery, M. L., Entenman, C., and Chaikoff, I. L., *Am. J. Physiol.*, **141**, 216 (1944).
9. Entenman, C., Chaikoff, I. L., and Montgomery, M. L., *J. Biol. Chem.*, **130**, 121 (1939).

THE EFFECT OF CASTRATION AND VARIOUS STEROIDS ON THE ARGINASE ACTIVITY OF THE TISSUES OF THE MOUSE*

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The demonstration that urinary androgen extracts (1, 2) and certain crystalline steroids (3–12) produce marked nitrogen retention in dogs and humans suggested that these steroids might affect enzymes concerned with protein metabolism. Herein is reported the effect of castration and a number of steroids on the arginase activity of several tissues of the mouse.

Methods

Pellets—The steroids[1] were made into cylindrical pellets of 14 ± 1 mg., diameter 2.7 mm., and height 2.7 ± 0.1 mm., by means of a hand press designed in this laboratory.

Animals[2]—Mice of the highly inbred Murray-Little dba strain were castrated under ether anesthesia at 16.0 to 19.5 gm. of body weight. They were fed Purina fox chow checkers.

In most of the experiments four or more mice were used. In a few instances, however, the scarcity of material limited the number of animals, but only obvious conclusions are made in these cases.

Duration of Experiments—The steroid pellets were implanted subcutaneously 30 days after castration. There were two series of experiments: (*a*) treatment for 10 days and (*b*) treatment for 30 days. All of the compounds were tested in the 30 day experiments but only the more active compounds in the 10 day experiments. Testosterone was tested also in a 20 day period.

Preparation of Tissues—The mice were fasted for 24 hours before autopsy.

They were killed by decapitation and exsanguination. The organs, except the small intestine, were removed, weighed, and dropped into heavy walled test-tubes containing 5 ml. of ice-cold redistilled water.[3] The small intestine was removed and placed in a Petri dish containing physiological salt solution, where it was slit lengthwise, freed of its contents, and washed twice with fresh portions of saline. Then the organ was cut into lengths of 5 to 10 mm. to facilitate homogenizing, blotted on filter paper, weighed, and dropped into the test-tube. The tissues were ground to a fine emulsified state in an all-glass apparatus similar to that used by Potter and Elvehjem (13). Then the homogenized tissues were diluted so that the arginase values would not exceed 12 units per ml. The liver and intestine were prepared first as stock mixtures containing 20 ml. of redistilled water per gm. of tissue. The liver mixture then was diluted further by pipetting 1 ml. into a 100 ml. volumetric flask containing 10 ml. of sodium barbital buffer[4] of pH 9.2 and making to volume with redistilled water. The intestine sample was diluted by adding 9 ml. of water to 1 ml. of the stock mixture. The kidney tissue was prepared in only one dilution of 40 ml. of water per gm. of tissue. 1 ml. of the final dilutions was used for each enzyme determination.

Arginase Determination—The arginase activity was determined by a modified combination of the Edlbacher and Rothler (15) and the Lightbody (14) methods. The entire procedure was carried out in a 25 × 200 mm. test-tube. The substrate was the same as that used by Lightbody (14) except that it was set at pH 9.2. The incubation was carried on for 6 hours in a water bath at 37°. The reaction was ended by the addition of 1 ml. of 0.3 N hydrochloric acid, which lowered the pH of the mixture to 7, and insertion of the test-tube in a boiling water bath for 5 minutes. The urea formed was determined by the urease-aeration method of Van Slyke and Cullen (16). The amount of ammonia derived from the tissue and substrate was determined by carrying out the above procedure without the incubation. The values were very small, 0.05 to 0.11 ml., 0.06 to 0.12 ml., and 0.07 to 0.21 ml. of 0.02 N hydrochloric acid for the liver, intestine, and kidney respectively. In order to determine the amount derived from the substrate alone, samples of the mixture were put through the complete procedure without the addition of the tissue. The values ranged from 0.00 to 0.08 ml. of 0.02 N hydrochloric acid. The arginase units were calculated from a curve prepared according to the procedure of Edlbacher and Rothler (15).

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[3] Redistilled water prepared in an all-glass still was used throughout the experiments.

[4] The sodium barbital increases the activity of the arginase (14). The same effect was noted under the above conditions.

Results

Distribution of Arginase—A study of the content of arginase in the several tissues of the mouse (Table I) indicates that the liver contains by far the greatest amount of the enzyme. The amount of arginase in the small intestine, though much less than that of the liver, is still quite large. The kidney contains relatively small amounts of the enzyme. The seminal vesicles and prostates contain small but definite quantities of the enzyme and the immature testes do not show any detectable amounts of enzyme. The distribution in the various tissues is in agreement with that reported by Edlbacher and Rothler (17) except for the amounts in the small intestine. These investigators reported only small amounts in this tissue.

Effect of Steroids on Liver and Intestine Arginase—None of the steroids

TABLE I

Arginase Content of Various Tissues of Mouse

The values are the average from eleven mice, about 110 days of age.

Tissue	Arginase		
		Standard deviation*	Ratio
	units per gm.		
Liver	11,200	±2500	100.00
Intestine	730	±85	6.52
Kidney	33	±6.8	0.30
Seminal vesicles and prostate	5	±0.9	0.04
Testes (immature)†	0		0.00

* $\sqrt{\Sigma(d^2)/(N-1)}$.

† Pooled testes of six mice, 30 to 45 days old.

had any effect on the arginase content of either the liver or intestine when calculated both for the total organ and per gm. of tissue. There were small differences but these changes were not statistically significant (*cf.* the standard deviations, Table I).[5] Therefore, the data for these tissues have been omitted.

Castration and Kidney Arginase—The amount of arginase per gm. of tissue is greater in the kidneys of the castrated than of the normal mice. The total amount of enzyme in the kidneys, however, is identical for both groups of animals. The decrease in kidney weight as a result of castration, therefore, is not accompanied by a decrease in arginase activity.

[5] In a preliminary report (18) increases in arginase activity of the liver and intestine of the rat were indicated; subsequent experiments have not supported this suggestion. The increase in the arginase of the kidney, however, has been confirmed.

TABLE II

Changes in Arginase Activity of Kidneys of Castrated Mice Treated for 30 Days with Pellets of Various Steroids

Steroid	No. of mice	Steroid absorbed	Kidney	Arginase* Total		Per gm.	
		mg	*mg*	*units*	*per cent differ-ence†*	*units*	*per cent differ-ence†*
17-Methyltestosterone‡	5	8.8	498	179 (161–195)	1278	359 (350–374)	632
Testosterone	6	8 3	539	182 (160–218)	1300	335 (311–364)	584
" propionate§	6	4 4	501	99 (89–111)	666	200 (183–257)	308
17-Methylandrostanediol-3(α),17(α)	7	2.6	521	94 (71–114)	624	181 (146–210)	269
Androstanol-17(α)-one-3	4	2.9	454	52 (49– 54)	300	115 (111–122)	135
α-Estradiol‖	5	2.7	316	28 (23– 32)	116	92 (85– 99)	88
Androstanediol-3(α)-17(α)	5	1.6	451	39 (30– 59)	200	84 (73–107)	71
17-Vinyltestosterone	8	8.1	401	31 (22– 54)	138	76 (59–101)	55
Testosterone acetate-3-propionate-17	2	1.3	374	25 (21– 29)	92	66 (58– 74)	35
Dehydroisoandrosterone	4	10.8	295	17 (14– 22)	31	58 (50– 72)	18
Androstenediol-3(β),-17(α)	4	0 6	249	13 (10– 15)	8	53 (39– 72)	8
Androsterone	3	3.8	301	16 (14– 18)	23	52 (50– 55)	6
Cistestosterone	2	3.6	257	14 (13– 14)	8	52 (50– 53)	6
Androstanedione-3,17	4	8.6	303	14 (13– 17)	8	49 (38– 60)	0
Castrate	20		257	13¶ (9– 17)		49** (32– 59)	
Pregnenol-3(β)-one-20	5	0 7	263	13 (11– 16)	0	48 (40– 58)	−2
3,17-Dimethylandrostadienol-17(α)	5	2.8	274	13 (10– 17)	0	48 (40– 54)	−2
3-Methylandrostadienol-17(α)	5	6.0	264	13 (7– 20)	0	47 (26– 67)	−4
Androstenedione-3,17	4	10 3	344	15 (11– 20)	12	43 (33– 55)	−12
17-Ethynyltestosterone††	6	0.5	275	11 (7– 17)	−15	42 (26– 68)	−14
Progesterone‡‡	5	5.6	273	12 (9– 17)	−8	42 (36– 60)	−14
17-Ethynylandrostenediol-3(β),17(α)	5	1.0	292	13 (8– 20)	0	42 (33– 52)	−14
Androstenediol-3(β),17(β)	3	2.1	255	11 (8– 13)	−15	41 (32– 47)	−16
Testosterone benzoate	4	0 3	268	11 (10– 13)	−15	41 (37– 47)	−16
Allopregnanol-3(α)-one-20	2	0.1	249	10 (9– 11)	−23	41 (35– 47)	−16

TABLE II—*Concluded*

Steroid	No. of mice	Steroid absorbed	Kidney	Arginase* Total			Arginase* Per gm	
		mg	*mg*	*units*		*per cent difference†*	*units*	*per cent difference†*
Etiocholanol-3(α)-one-17	1	7.9	242	10		−23	38	−22
Pregnanol-3(α)-one-20	2	2.6	260	10	(9– 10)	−23	37 (33– 40)	−24
17-Ethyltestosterone	9	5.1	346	13	(8– 19)	0	36 (23– 51)	−26
17-Ethynylandrostane-diol-3(β),17(α)	5	0 5	298	11	(8– 17)	−15	35 (26– 55)	−29
Isoandrosterone	6	9.4	264	9	(7– 12)	−31	33 (26– 39)	−33
Normal	11		386	13	(8– 19)	0	33 (25– 43)	−33
17-Methylandrostene-diol-3(β),17(α)	6	0.9	306	10	(8– 12)	−23	32 (28– 36)	−35
17-Methylandrostane-diol-3(β),17(α)	4	0 3	337	10	(7– 13)	−23	30 (20– 38)	−39
Androstanediol-3(β),-17(α)	3	0 2	287	8	(7– 8)	−38	25 (23– 26)	−49

* The figures in parentheses indicate the range of values.

† Change from castrate values   The figures are the per cent of the averages.

‡ Metandren.

§ Perandren

‖ Ovocylin

¶ Standard deviation, $\sqrt{\Sigma(d^2)/(N-1)} = \pm 2.1$.

** Standard deviation, $\sqrt{\Sigma(d^2)/(N-1)} = \pm 7.7$.

†† Lutocylol, anhydrohydroxyprogesterone, or pregnenynolone.

‡‡ Lutocylin.

Effect of Steroids on Kidney Arginase—Many of the steroids have an effect on the arginase content of the kidney (Tables II and III).   The very small amount of enzyme normally present in this organ is increased as much as 1300 per cent for total amount and 632 per cent per gm. of tissue.

Arginase Activity and Kidney Size[6]—Increases in arginase activity occur only in those kidneys that have increased in size as a result of steroid treatment[7] (Tables II and III).   There is, however, no direct correlation of kidney weight with increased arginase activity.   Methyltestosterone, testosterone, testosterone propionate, and 17-methylandrostanediol-3(α),17(α) produce large and similar increases in kidney size.   The arginase activities, however, are very different.   The first two steroids are twice as potent as the other two compounds (Table II, Fig. 1).

[6] The effect of the steroids on kidney weight will be discussed in another report.

[7] Testosterone propionate does not increase the kidney weight of the rat but markedly increases the arginase content (Kochakian, unpublished).

The results after α-estradiol treatment are very interesting. This compound produces only a small increase in kidney size; yet it causes a relatively large increase in arginase activity. This is especially true of the mice treated for only 10 days. The increase in enzyme activity for the kidneys of these mice is more than twice that of the animals treated 30 days (Fig. 1) and the same as that for the mice treated for 10 days with the more potent renotropic steroid, testosterone propionate. The effect of α-estradiol becomes of greater significance when it is noted that many compounds produce kidneys of greater size or similar size with either a smaller effect, no effect, or even a decrease (Tables I and II) in arginase activity.

Duration of Treatment and Arginase Activity—In order to determine whether the effect on arginase activity required the prolonged treatment

TABLE III

Changes in Arginase Activity of Kidneys of Castrated Mice Treated for 10 Days with Pellets of Various Steroids

Steroid	Mice	Steroid absorbed	Kidney	Arginase*			
				Total		Per gm.	
		mg	*mg*	*units*	*per cent difference†*	*units*	*per cent difference†*
Testosterone	6	3.2	432	127 (106–152)	876	289 (237–364)	490
Methyltestosterone	3	3.1	411	112 (101–132)	760	273 (248–330)	457
Testosterone propionate	3	1 7	409	59 (53– 62)	354	144 (139–152)	194
α-Estradiol	4	0 5	315	45 (40– 54)	246	143 (120–151)	192
Androstanediol-3 (α),17 (α) ..	3	0.6	349	27 (25– 29)	108	79 (65– 89)	61
Androstanol-17 (α)-one-3.	4	0.7	368	23 (16– 32)	77	62 (44– 86)	27

* The figures in parentheses indicate the range of values.

† Change from castrate values. The figures are the per cent of the averages.

with the steroids, experiments were run in which the mice were treated for only 10 days and in one instance also for 20 days. The results are listed in Table III and are compared with the 30 day experiments in Fig. 1.

All of the steroids except androstanol-17(α)-one-3 show a greater rate of response at the shorter period of treatment. In addition α-estradiol shows a greater total response at the 10 day period than at the 30 day period in spite of the fact that the kidney weights in the two periods of treatment are the same. This decrease in effect after the longer period of treatment is probably related to the toxic properties of estrogens. These mice lost weight.

Amount of Steroid Absorbed and Arginase Activity—There is no general correlation of amount of steroid absorbed with arginase activity. Dehy-

droisoandrosterone, androstenedione-3,17, isoandrosterone, androstane-
dione-3,17, 17-vinyltestosterone, and etiocholanol-3(α)-one-17 are ab-
sorbed in greater or similar amounts to methyltestosterone and testosterone;
yet these compounds produce either small changes or no changes in arginase
activity of the kidneys. However, when only those four compounds that
produce maximum kidney size are considered, there seems to be a relation-
ship between amount of steroid absorbed and increase in arginase activity.
Methyltestosterone and testosterone show similar values for steroid ab-
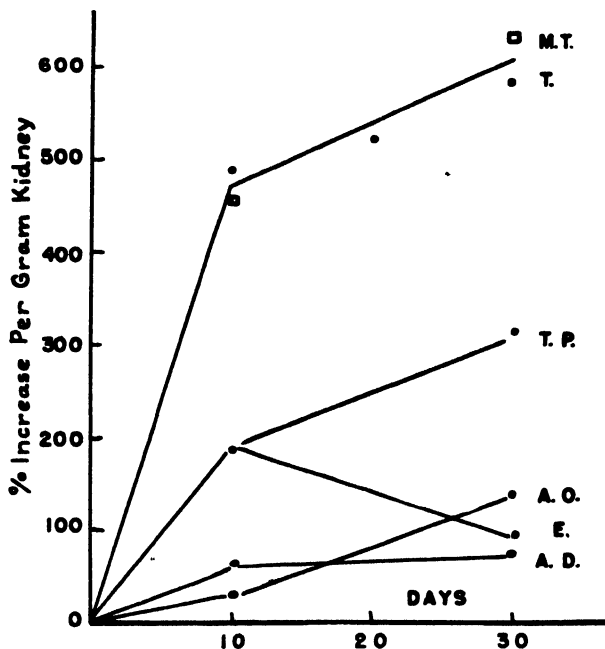
FIG 1. Effect of steroids on kidney arginase. M. T , methyltestosterone; T.,
testosterone; T. P., testosterone propionate; A. O., androstanol-17(α)-one-3; E.,
α-estradiol; A D , androstanediol-3(α),17(α).

sorbed, kidney weight, and arginase activity. Testosterone propionate and
17-methylandrostanediol-3(α),17(α) are absorbed in smaller amounts than
the former compounds and also show a smaller increase in arginase activity.[8]

Chemical Structure and Effect on Arginase Activity—The most potent

[8] The implantation of two pellets of testosterone propionate to provide an amount
of material nearly equivalent to that absorbed from the testosterone pellets gave
arginase values similar to those obtained with testosterone without any further in-
crease in kidney size These results will be published in detail when similar experi-
ments have been completed with the other compounds

compounds are methyltestosterone and testosterone. The saturation of the double bond in testosterone to give androstanol-17(α)-one-3 causes a marked decrease in the ability of the latter compound to increase kidney arginase. Conversion of the ketone group to an α-hydroxy group as in androstanediol-3(α),17(α) produces a further decrease in arginase-stimulating property. Moreover, it must be concluded that the presence of the 17-α-hydroxy group seems to be essential because androstenedione-3,17, androstanedione-3,17, and androsterone have no effect on arginase activity.

Stereoisomerism in the molecule plays an important rôle. This is most clearly shown in a comparison of the effects of testosterone and its 17-hydroxy isomer, cistestosterone. Testosterone is one of the most potent steroids in producing increased arginase activity, while cistestosterone has no effect. The influence of the spatial arrangement of the hydroxyl groups is further demonstrated by a comparison of the potencies of 17-methylandrostanediol-3(α),17(α), and androstanediol-3(α),17(α) with their 3-hydroxy stereoisomers, 17-methylandrostanediol-3(β),17(α) and androstanediol-3(β),17(α). Apparently neither the 3(β)- nor the 17(β)-hydroxy group nor both together favor increases in arginase activity.

The addition of alkyl groups in the 17 position has varied effects on arginase activity. Although methyltestosterone is no more potent than testosterone, 17-methylandrostanediol-3(α),17(α) is about 4 times as potent as androstanediol-3(α),17(α). The addition of the next higher alkyl group (ethyltestosterone) results in a complete disappearance of the arginase-stimulating property. If a double bond is introduced into the ethyl group (17-vinyltestosterone), there is a partial restoration of the arginase-increasing property which disappears again when the double bond is replaced by a triple bond to give 17-ethynyltestosterone. Furthermore, the introduction of the methyl group into the 3, 17, or both positions, or the ethynyl group into the 17 position of inactive compounds does not increase arginase activity. In fact, 17-methylandrostenediol-3(β),17(α) and 17-methylandrostanediol-3(β),17(α) bring about a decrease in the arginase activity.

Esterification—Both the ester and enol diester of testosterone stimulate much smaller increases in the kidney arginase than the unesterified compound. The effect of testosterone propionate is only about 50 per cent and that of testosterone acetate-3-propionate-17 about 6 per cent that of testosterone, and testosterone benzoate is completely ineffective. These decreases are due, at least in part, to the decreased absorption of the esterified steroids (*cf.* foot-note 8).

Cause of Increase in Arginase Activity—Since it was known that many factors enhance the activity of arginase, several experiments were run to determine the general nature of the induced enhancement of enzyme action. The addition of 0.5 mg. of testosterone to duplicate samples of incubation

mixture resulted in no significant change (−4.5 per cent). The mixing in equal amounts of homogenized kidney tissue from non-treated animals and animals treated with methyltestosterone or testosterone resulted in a simple summation of the respective activities (Table IV). A similar result was obtained when kidney samples of mice treated with testosterone and α-estradiol were mixed. As a further test a mixture of a non-treated and a testosterone-treated kidney sample was run without the activator, cobaltous chloride. As was to be expected, there was a decrease in the activity of the individual samples, owing to the omission of the cobaltous chloride, but the mixture decreased proportionately. Therefore, it must be concluded from the summation of activities in all of the above experiments that the increases

TABLE IV

Concerning Cause of Increase in Kidney Arginase Activity after Treatment with Steroids

Treatment		Arginase		
		Average	Mixture*	Difference
	units	*units*	*units*	*per cent*
Methyltestosterone	5.03	5 62	5.49	−2 3
Castrate	0 59			
Testosterone	4.21	4.84	4.40	−9.1
Castrate	0 63			
Testosterone	3 92	4.60	4.20	−8.7
Castrate	0.68			
Testosterone	2 81†	3.26	3.00†	−8.0
Castrate	0.45†			
Testosterone	5 25	7.09	7.13	+0.6
α-Estradiol	1.84			

* The two samples of homogenized kidney tissue were mixed in equal quantities.
† The same samples as for the preceding test, but no cobaltous chloride activator was added to the substrate

in kidney arginase activity noted after treatment with the various steroids are not due to the production of an activator but to the actual production of more arginase.

DISCUSSION

The increase in kidney arginase noted after treatment with many of the steroids is not for the formation of greater amounts of urea by the Krebs-Henseleit cycle (19, 20). The same steroids that produce the greatest increases in enzyme activity also cause a decrease in urinary (1–3) and blood urea (1–3). It is impossible at present, however, to state the specific purpose of the large increases in arginase activity. It is known that the arginase in the kidney is localized in the proximal convoluted tubules (21).

Therefore, the observed enlargement of this part of the kidney after steroid treatment (22, 23) is probably, in part, to produce greater amounts of arginase to satisfy metabolic demands imposed upon the kidney by the steroids involved. Furthermore, the lack of correlation of the increase in arginase activity with any of the other known properties of the steroids indicates that it is a separate phenomenon.

The inability of any of the steroids, especially those compounds that are known to stimulate protein anabolism, to change the liver arginase must be interpreted to mean that those amino acids which provide ammonia to be used in the Krebs-Henseleit cycle (19, 20) for urea formation are not affected by these steroids. The observed protein anabolic effects must be through other amino acids. Furthermore, the mechanism for the protein anabolic property of the anterior pituitary growth hormone must be different from that of the steroids, since this factor is reported to decrease liver arginase (24). This does not exclude the possibility that the anterior pituitary growth hormone works through the same mechanism as the androgens in addition to some other mechanism or mechanisms.

SUMMARY ·

Mice weighing 16.5 to 19.5 gm. were castrated, and 1 month later 14 $\pm$ 1 mg. pellets of various steroids were implanted subcutaneously. Arginase determinations were made 10 and 30 days later. None of the steroids affected the enzyme content of the liver or intestine, but many of these compounds markedly increased and a few decreased the arginase content of the kidneys. The order of change in per cent difference per gm. of kidney tissue for the 30 day experiments was as follows: methyltestosterone 632, testosterone 584, testosterone propionate 308, 17-methylandrostanediol-3(α), 17(α) 269, androstanol-17(α)-one-3 135, α-estradiol 88, androstanediol-3(α),17(α) 71, 17-vinyltestosterone 55, testosteroneacetate-3-propionate-17 35. Much greater changes were obtained when the values were calculated on the basis of total tissue. Eighteen other compounds had no effect and isoandrosterone, 17-methylandrostenediol-3(β),17(α), and 17-methylan-drostanediol-3(β),17(α) caused decreases of 33 to 39 per cent. The changes were not related to changes in kidney weight or amount of material absorbed.

The increases in arginase activity represented greater amounts of enzyme and not a production of arginase activators.

The kidneys of the castrated mice contained the same amount of total arginase but greater amounts per gm. of tissue than did those of the normal mice.

BIBLIOGRAPHY

1. Kochakian, C. D., *Proc. Soc. Exp. Biol. and Med.*, **32,** 1064 (1935).
2. Kochakian, C. D., and Murlin, J. R., *J. Nutr.*, **10,** 437 (1935).
3. Kochakian, C. D., and Murlin, J. R., *Am. J. Physiol.*, **117,** 642 (1936).
4. Thorn, G. W., and Engel, L. L., *J. Exp. Med.*, **68,** 299 (1938).
5. Gaebler, O. H., and Tarnowski, S. M., *Endocrinology*, **33,** 317 (1943)
6. Kenyon, A. T., Sandiford, I., Bryan, A. H., Knowlton, K , and Koch, F. C., *Endocrinology*, **23,** 135 (1938).
7. Kenyon, A. T., Knowlton, K., Koch, F. C., and Lotwin, G., *Endrocrinology*, **26,** 26 (1940).
8. Albright, F., Parson, W., and Bloomberg, E., *J. Clin. Endocrinol.*, **1,** 375 (1941).
9. Kenyon, A. T., Knowlton, K., Lotwin, G., and Sandiford, I., *J. Clin. Endocrinol.*, **2,** 690 (1942).
10. Knowlton, K., Kenyon, A. T., Sandiford, I., Lotwin, G., and Fricker, R., *J. Clin. Endocrinol.*, **2,** 671 (1942).
11. Basset, S. H., Keutmann, E. H., and Kochakian, C. D , *J. Clin. Endocrinol.*, **3,** 400 (1943).
12. Albright, F., *Harvey Lectures*, **38,** 123 (1942-43).
13. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114,** 495 (1936).
14. Lightbody, H. D., *J. Biol. Chem.*, **124,** 169 (1938).
15. Edlbacher, S., and Rothler, H., *Z. physiol. Chem.*, **148,** 264 (1925).
16. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19,** 211 (1914).
17. Edlbacher, S., and Rothler, H., *Z. physiol. Chem* , **148,** 273 (1925).
18. Kochakian, C. D., and Clark, L. C., Jr., *J. Biol. Chem.*, **143,** 795 (1942).
19. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210,** 33 (1932).
20. Krebs, H. A., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **3,** 247 (1934).
21. Weil, L., and Ely, J. O., *J. Biol. Chem.*, **112,** 565 (1935-36).
22. Selye, H., *J. Urol.*, **42,** 637 (1939).
23. Pfeiffer, C., Emmel, V., and Gardner, W. U., *Yale J. Biol. and Med.*, **12,** 493 (1940).
24. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. E., *Am. J. Physiol.*, **138,** 438 (1943).

INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XVII. THE DETERMINATION OF GLUTAMIC ACID IN PROTEIN HYDROLYSATES BY A MICROBIOLOGICAL METHOD*

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The microbiological determination of arginine, isoleucine, leucine, tryptophane, and valine in the hydrolysates of casein and other proteins has been described recently by McMahan and Snell (2), Kuiken *et al.* (3), Greene and Black (4), and Hegsted (5). The microorganisms *Lactobacillus arabinosus* 17-5 and *Lactobacillus casei* and a turbidimetric or titrimetric method of analysis were employed. It was reported that negligible amounts of isoleucine, leucine, and valine were lost because of humin formation even in the presence of carbohydrates and that only the naturally occurring isomers of these amino acids were active. The values under varying hydrolytic conditions and at different levels of protein hydrolysate agreed closely, the recovery of amino acids added to protein hydrolysates was nearly theoretical, and the percentage of amino acids agreed satisfactorily with values selected from the literature. Data of comparable precision and accuracy have been obtained in analogous experiments performed in the writers' laboratory.[1] It seems evident from these findings that amino acids in protein hydrolysates may be determined by microbiological assay conveniently and, for many purposes, with satisfactory accuracy.

It could have been predicted that greater difficulties would be encountered in the determination of some amino acids than of others in protein hydrolysates because of the stimulatory or inhibitory influence exerted by amino acids or other hydrolytic products. It is of interest in this connection that Hutchings and Peterson (8) have shown that histidine and isoleucine were inhibitory, lysine and alanine were stimulatory, and that

[1] The following percentages (corrected for moisture and ash) of amino acids in two samples of casein, one prepared by the present method and the other by the Van Slyke and Bosworth (6) procedure, were reported (7) at the meeting of the American Chemical Society, Cleveland, April 6, 1944: arginine 3.4, 3.4, isoleucine 5.9, 6.3, leucine 9 1, 9.1, methionine 3.1, 3.2, tyrosine 5.2, 4.8, and valine 6.8, 7.3.

these effects were roughly additive in respect to the growth of *Lactobacillus casei*.

Although it has been found that glutamic acid is essential for the growth of *Lactobacillus arabinosus* and *Lactobacillus casei*, assays of this amino acid have not been satisfactory. The values for the percentage of this amino acid in casein obtained initially with *Lactobacillus arabinosus* 17-5 were very much higher than the commonly accepted figure, even though the standard curve was reproducible and of a type which had been shown to be satisfactory for the assay of glutamic acid as well as of other amino acids in a simple test mixture (9). After investigation of the effect of additional amino acids and different levels of components, a basal medium was derived with which a standard curve of increased slope was obtained. This curve yielded dependable data for glutamic acid. The original medium was modified by increasing 10 times the levels of adenine, guanine, uracil, and the vitamins, and by adding supplements of folic acid, proline, hydroxyproline, serine, norleucine, and glycine. Similar observations have been made by McMahan and Snell (2) who stated that growth of this microorganism was heavier and antagonistic effects were minimized when nearly complete, rather than restricted, mixtures of amino acids were used.

EXPERIMENTAL

Casein was prepared by adding 0.5 N hydrochloric acid slowly with stirring to a mixture of 1400 ml. of skim milk[2] and 9800 ml. of distilled water contained in a 12 liter flask. The pH of the final mixture in the seven runs ranged from 4.75 to 4.88 and averaged 4.83. The suspension was filtered, and the casein was washed repeatedly by suspending it in distilled water and stirring the mixture. The final washings contained neither chloride nor phosphate ion. The purified casein was triturated successively with 95 per cent ethanol, absolute ethanol, and absolute diethyl ether. The yield of pure white, air-dried product was 198 gm. from 9800 ml. of skim milk or 20.2 gm. per liter.

This product contained 6.21 per cent moisture,[3] 0.55 per cent ash, 0.59 per cent phosphorus,[4] and 15.60 per cent nitrogen[5] (corrected for moisture and ash).

[2] Skim milk obtained through the courtesy of the Edgemar Farms dairy from Holstein cows was chilled immediately and preserved in a refrigerator.

[3] The constant weight observed when a sample of the casein was dried at 65° in a partial vacuum in an Abderhalden drier was unchanged after the material was dried for an additional 24 hours at 103°.

[4] Determined by nitric acid oxidation (10) of casein and colorimetric analysis of phosphate (11). The percentage of phosphorus is considered to be accurate within

Silk fibroin was prepared by heating a suspension of silk waste in distilled water until all soluble material had been removed and the fibers attained a constant dry weight. This procedure, described by Fischer and Skita (12), has been employed by nearly all workers since earliest times.[6] It seems probable that most of the sericin is removed by this treatment. In the present experiments 100 gm. of silk waste[7] suspended in 1500 ml. of distilled water were heated for 2 hours in an autoclave at about 120°. The dark gray liquid was decanted, and the fibers were washed thoroughly with distilled water. After this process was repeated four times, the yield of fibers dried at 55° was 71.7 gm. This weight was unchanged by an additional treatment. Fischer and Skita (12) found 68.5 per cent of fibroin
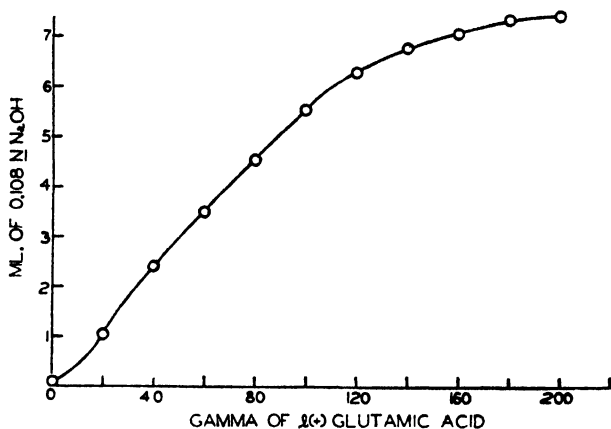
FIG. 1. Standard curve for the assay of $l(+)$-glutamic acid with *Lactobacillus arabinosus* 17-5   The curve shown was derived from a triplicate assay. Maximum variations in the titration values are denoted by the diameter of the circles.

in silk, but Vignon (14) reported that different silks contained from 70.1 to 82 per cent of fibroin.

±10 per cent, since recoveries averaging 109 and 92 per cent, respectively, were obtained with mixtures containing approximately 100 and 165 mg. of casein and quantities of c p KH_2PO_4 equivalent to 0.3 and 0 6 mg of phosphorus. Van Slyke and Bosworth (6) found 0 71 per cent of phosphorus in casein, but they did not report the accuracy of this value.

[5] Determined by semimicro-Kjeldahl procedure   Approximately 92, 54, and 109 mg samples of casein were digested for 20 hours with 5 ml. of concentrated sulfuric acid solution containing 0.5 gm. of powdered selenium, 250 gm. of $KHSO_4$, and 5 gm of anhydrous $CuSO_4$ per liter. The uncorrected values were 14.50, 14.44, 14 54 per cent nitrogen.

[6] The older literature has been reviewed by Shelton and Johnson (13).

[7] Italian frisons in the gum obtained from Cheney Brothers Manufacturers, South Manchester, Connecticut

<div align="center">TABLE I</div>

Basal Medium for Assay of Glutamic Acid with Lactobacillus arabinosus 17-5

The hydrochloric acid solutions of the amino acids, purines, and pyrimidine were mixed, about 200 ml. of distilled water were added, and the indicated quantities of dextrose and sodium acetate were dissolved in this solution. 5 ml. each of the vitamin solution, the folic acid solution, and Salts A and B were added. The final solution was neutralized to pH 6.8 with sodium hydroxide, diluted to 500 ml., and, if not utilized immediately, preserved overnight in the refrigerator.

Amino acids*		*mg.*			*gm.*
	$l(-)$-Tyrosine	20	Dextrose		10.0
	dl-Serine	50	Sodium		6.0
	$l(-)$-Tryptophane	50	acetate		
	$l(+)$-Arginine monohydro-	50			
	chloride				
	$l(-)$-Histidine monohy-	50			
	drochloride monohydrate				*mg.*
	dl-Methionine	100	Salts A‡	KH$_2$PO$_4$	500
	$l(-)$-Cystine	100		K$_2$HPO$_4$	500
	dl-Phenylalanine	100			
	Glycine	100			
	$l(-)$-Proline	100			
	$l(-)$-Hydroxyproline	100	Salts B§	MgSO$_4$·7H$_2$O	200
	dl-Norleucine	100		Fe$_2$(SO$_4$)$_3$·6H$_2$O	10
	dl-Threonine	200		MnSO$_4$ 4H$_2$O	10
	$l(-)$-Leucine	200			
	dl-Isoleucine	200			
	dl-Valine	200			
	dl-Lysine monohydro-	200	Vitamins‖	Thiamine hy-	1.0
	chloride			drochloride	
	dl-Alanine	200		Pyridoxine	1.6
	Asparagine, natural	400		dl-Calcium	2.0
				pantothenate	
Purines				Riboflavin	2.0
and pyr-				Niacin	2.0
imidine†	Adenine sulfate	100		Biotin	0.005
	Guanine hydrochloride	100		p-Aminobenzoic	0.100
	Uracil	100		acid	
				Folic acid¶	0.004

* The quantities of amino acids indicated were dissolved in about 75 ml of 2 N hydrochloric acid.

† The quantities indicated were dissolved in about 20 ml of 2 N hydrochloric acid.

‡ A stock solution was prepared containing 10.0 gm. of each salt per 100 ml. of distilled water

§ A stock solution was prepared containing 10.0 gm. of MgSO$_4$·7H$_2$O, 0.50 gm. of Fe$_2$(SO$_4$)$_3$·6H$_2$O, and 0.50 gm. of MnSO$_4$·4H$_2$O per 250 ml. of N hydrochloric acid.

‖ A stock solution was prepared containing 50 mg. of thiamine hydrochloride, 80 mg. of pyridoxine, 100 mg. of dl-calcium pantothenate, 100 mg. of riboflavin, 100 mg. of niacin, 5 mg of p-aminobenzoic acid, and 53 ml. of a 50 per cent ethanol solution

TABLE I—*Concluded*

containing 4.8 γ per ml. of crystalline biotin per 250 ml. of 50 per cent ethanol. The pH of this solution was 3.

¶ Prepared from liver powder 1:20, obtained from The Wilson Laboratories, by norit adsorption and elution according to the procedure of Hutchings *et al.* (61). The solution contained 0.77 γ of folic acid per ml.

TABLE II

Results of Assay of Glutamic Acid in Casein Hydrolysate

Five mixtures each containing 0 500 gm. of casein and 2.5 ml. of 8 N hydrochloric acid were refluxed simultaneously for 43½ hours in the same heating bath.

Casein per tube	Titration volume of 0 108 N NaOH per tube	Glutamic acid* found	
		Per tube	Casein
γ	*ml*	γ	*per cent*
100	1.05	20.6	20.6
100	0.97	19.2	19.2
200	2.45	42.9	21.5
200	2.46	43.0	21.5
300	3.75	64.7	21.6
300	3.79	65.3	21.8
400	4.73	83.5	20.9
400	5.08	90.6	22.6
500	5.84	108.0	21.6
500	5.98	111.5	22.3

* The average mean deviation from the mean of the values at different levels was 0.56 or 2.7 per cent. The percentages of glutamic acid found in the five samples of casein were 20.4, 21.2, 21.3, 21 4, and 21.1. The average percentage is 21 1 for the five samples and 21.25 for the last four. All percentages are uncorrected for moisture and ash. The uncorrected percentages of glutamic acid found in preliminary experiments after the refluxing times in hours (shown in the parentheses) were 15 5 (½), 20.1 (14), 22.9 (14), 23.2 (14), 19.9 (16), and 23.6 (16)

TABLE III

Results of Assay of Glutamic Acid in Silk Fibroin Hydrolysate

Silk fibroin per tube	Titration volume of 0.0633 N NaOH per tube	Glutamic acid* found	
		Per tube	Silk fibroin
γ	*ml.*	γ	*per cent*
1250	3.62	26.0	2.08
1250	3.49	24 9	1.99
2500	6.29	51.0	2.04
2500	6.02	48.4	1.94
3750	8.25	74.6	1.99
3750	8.28	75.0	2.00
5000	9.97	101.2	2.02
5000	10.25	106.9	2.14

* The average value for glutamic acid in silk fibroin was 2 03 ± 0 05 per cent, uncorrected, and 2.16 per cent, corrected for moisture and ash. The average mean deviation from the mean of the experimental values is 0.048 or 2.34 per cent.

TABLE IV

Composition of Amino Acid Test Mixture Simulating That of Casein

Amino acid	Amount in mixture	Natural form in		
		Mixture	Total natural form in mixture	Casein (literature)
	mg.	*per cent*	*per cent*	*per cent*
dl-Alanine . ..	26.4	0.93	1.34	1.9 (18)
l(+)-Arginine monohydrochloride ..	44.6	2.60	3.77	3.72 (19)
Asparagine, natural	59.4	4.19	6.04	5.95* (20)
l(−)-Cystine	3.9	0.28	0.40	0.30 (21)
l(+)-Glutamic acid	220.1	15.5	22.4	22.0 (22)
Glycine.	4.4	0.31	0.45	0.45 (18)
l(−)-Histidine monohydrochloride monohydrate	33 9	1.77	2.56	2.50 (23)
l(−)-Hydroxyproline	2.1	0.15	0.21	0.23 (23)
dl-Isoleucine	126.3	4 46	6.45	6.0 (3)
l(−)-Leucine	92.2	6.50	9.39	9.3 (3)
dl-Lysine monohydrochloride	154.4	4.36	6.31	6.25 (24)
dl-Methionine	66.5	2.34	3.39	2.87 (25)
dl-Phenylalanine	78.6	2.77	4.00	5.8 (26)
l(−)-Proline	82.8	5.84	8.44	8.7 (27)
dl-Serine	100.6	3.54	5.12	5.0 (28)
dl-Threonine ..	69.4	2.45	3 54	3.5 (29)
l(−)-Tryptophane	14.9	1.05	1.52	1.15 (4)
l(−)-Tyrosine	53.8	3.79	5.47	5.36 (30)
dl-Valine	158.4	5 57	8.05	6.7 (2)
Ammonium chloride.	25.0	0.69†	0.99†	1.61† (23)
Total	1417 7	69.1	99.84	99.29

* Aspartic acid.

† Calculated as NH_3.

The product contained 5.68 per cent moisture,[8] 0.25 per cent ash, and 18.7 per cent nitrogen[9] (corrected for moisture and ash). The specific rotation[10] in 12 N hydrochloric acid was −50.45°.

[8] Sample dried to constant weight at 65° in an Abderhalden drier.

[9] Determined by digesting nine 85 to 225 mg samples of silk fibroin for 18 hours with the same sulfuric acid solution that was employed with casein. The uncorrected values were 17 61, 17 68, 17 67, 17 55, 17 64, 17.67, 17.58, 17.54, and 17.62 per cent nitrogen.

[10] A sample of 0 3896 gm of fibroin was dissolved in 140 ml of 12.03 N hydrochloric acid, the mixture was filtered after about 10 minutes, and the rotation of the clear violet-colored solution was measured in sodium light at 23° after about 55 minutes The specific rotation was −50 45°   Values ranging from −39 40° to −50.00° determined essentially under the same conditions were reported by Vignon (14, 15) for different silks, and Harris and Johnson (16) found −43.98°

TABLE V

Results of Assay of Glutamic Acid in Amino Acid Test Mixture

Amino acid mixture per tube	Titration volume of 0 108 N NaOH per tube	Glutamic acid*	
		Found per tube	Found
γ	*ml.*	γ	*per cent*
141.77	1.12	21.3	15.0
141.77	1.09	21.0	14.9
283.54	2.52	44.0	15.5
283.54	2.59	45.0	15.9
425.31	3.94	68.1	16.0
425.31	3.96	68.6	16.1
567.08	5.18	92.8	16.4
567.08	5.14	91.8	16.2
708.85	6.12	115.3	16.3
708.85	6.14	116.0	16.4

* The average value for glutamic acid found was 15.87 ± 0.4 per cent, and the average mean deviation from the mean of the experimental values was 0.62 or 2.76 per cent. The average value found is 102.3 per cent of 15.52, the percentage of glutamic acid present in the mixture   The values 15.9 and 15.9 were found for glutamic acid in the amino acid mixture after intervals of 7 and 31 days, respectively. **There was no apparent change in such a mixture made up in 3 N hydrochloric acid and stored in the refrigerator for 1 month**

TABLE VI

Recovery of Glutamic Acid from Protein Hydrolysates

Protein hydrolysate	Glutamic acid				
	Added per ml	Found per ml.	Present from hydrolysate per ml	Recovered	
	γ	γ	γ	γ	*per cent*
Casein	10.0*	19.98†	9.97	10.01	100.1
Silk fibroin .	11 25‡	23.8§	12.7	11.1	99.7

‡ 20.0, 30.0, 40.0, and 50.0 γ of glutamic acid per tube were added in duplicate to a hydrolysate containing 100.0, 150 0, 200 0, and 250 0 γ of casein per tube.

† The glutamic acid found ranged from 40.0 to 100.0 γ per tube and 19.0 to 20.6 γ per ml

‡ 11.25, 22.50, 33.75, 45 00, and 56.25 γ of glutamic acid per tube were added in duplicate to a hydrolysate containing 625, 1250, 1875, 2500, and 3125 γ of fibroin per tube.

§ The glutamic acid found ranged from 25.1 to 121.0 γ per tube and 22.2 to 25.8 γ per ml.

The microorganism *Lactobacillus arabinosus* 17-5 and the assay technique described previously were employed.[11]   The amino acids were high purity

[11] The suggestion of McMahan and Snell (2) that the assay tubes may be covered with toweling instead of plugged with cotton was found to be entirely satisfactory.

products obtained from Merck and Company and from Amino Acid Manufacturers. The desirability of using highly purified amino acids is emphasized by the recent report of Hegsted and Wardwell (17) that some lots of *dl*-leucine contained significant amounts of isoleucine.

The composition of the basal medium is given in Table I, and a typical standard curve is shown in Fig. 1. The results of the assay of glutamic acid in casein hydrolysates, silk fibroin hydrolysates, and an amino acid test mixture of a composition simulating that of casein are shown in Tables II to VI.

<div align="center">DISCUSSION</div>

It would appear from the experimental evidence presented that data of relatively high precision are attainable in the assay of glutamic acid in the acid hydrolysates of casein and silk fibroin. Because of the wide difference in the amino acid composition of these proteins, it seems probable that the procedure described may be applied as satisfactorily to the assay of glutamic acid in the hydrolysates of other proteins. There has long been urgent need for a more satisfactory method for the determination of glutamic acid in view of the importance of this amino acid, both in plants and animals, and the inconvenience or inaccuracy of classical procedures.[12]

[12] Glutamic acid has been commonly determined by isolation as its hydrochloride. This procedure, devised by Hlasiwetz and Habermann (31) in 1873, was employed extensively by Osborne and many other workers as a preliminary step in the determination of amino acids by the ester method. In 1914, Foreman (32) pointed out that it is more difficult to isolate glutamic acid from some protein hydrolysates than from others and that the percentages reported previously for this amino acid were unreliable. Foreman found 21.77 per cent of glutamic acid in casein, based on an analysis of the mixture of glutamic and aspartic acids obtained from the ethanol-insoluble calcium salts of these amino acids and of the amino nitrogen before and after hydrolysis of the pyrrolidonecarboxylic acid in the gummy residue. The sample of Hammarsten's casein contained 14.08 per cent nitrogen, uncorrected, and 15.35 per cent, corrected for 9.03 per cent moisture. In 1918, Dakin (27) obtained 20.8 (21.6, 21.2, and 19.6) per cent of glutamic acid isolated as the analytically pure hydrochloride from the amino acid fraction of a casein hydrolysate not extracted by *n*-butyl alcohol. Foreman's value of 21.8 per cent glutamic acid in casein was confirmed by Melville (33) who boiled the hydrolysate at pH 3, extracted the pyrrolidonecarboxylic acid with ethyl acetate, hydrolyzed it with hydrochloric acid, and isolated the glutamic acid as its hydrochloride This procedure was modified by Pucher and Vickery (34) for the determination of glutamine in plant tissues by measuring the amino nitrogen before and after hydrolysis of the pyrrolidonecarboxylic acid. A simplified pyrrolidonecarboxylic acid-amino nitrogen procedure has been described recently by Olcott (35) who found 22.0 per cent (20.5 to 24.7, range in nineteen determinations) in a sample of commercial casein containing 14.0 per cent nitrogen. Since recalculation on the basis of 15.7 per cent nitrogen gave approximately 25 per cent glutamic acid, it was suggested that this figure may have been too high, perhaps as much as 10 per cent, because of intrinsic errors which would tend to increase the

The value 22.00 reported in 1943 by Chibnall and coworkers (22) as the percentage of glutamic acid in casein is thought to be more dependable than any other. Unfortunately, the procedure employed is tedious, time consuming, and difficult. If the accuracy of this figure, considered probable by these workers, is accepted, we may assume that the percentage of glutamic acid in casein is 22.0 ± 0.5. On this basis the figures quoted by Foreman (32), Dakin (27), Melville (33), and Macara and Plimmer (36) are correct within the indicated limit of experimental error.

It was found in the present experiments that casein contains 22.5 per cent of glutamic acid. Because it seemed probable that this figure, the maximum value in accord with the data and conclusions of Chibnall and his associates, is nearly the correct one, the following experiment[13] was per-

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apparent glutamic acid content   In 1940, Macara and Plimmer (36) found 22.3 (21.6 and 22 9) per cent of glutamic acid in a sample of casein dried at 105° and containing 15 08 per cent nitrogen, 0.82 per cent phosphorus, and 1.10 per cent ash. Glutamic acid was determined by the method of Cohen (37)   The β-cyanopropionic acid resulting from the oxidation of glutamic acid with chloramine-T is hydrolyzed to succinic acid, which is extracted with ether, dissolved in citrate buffer, and estimated by determining the oxygen uptake under the action of succinic dehydrogenase in a Warburg apparatus   In 1943, Chibnall and coworkers (22) reported the values 22.00 and 22 00 for the percentage of glutamic acid in two samples of casein prepared by the method of Cohn and Hendry (38) and containing 15.73 (15.72 to 15.75, range of eight determinations) per cent nitrogen corrected for 0.75 per cent ash and 10.2 per cent moisture. These workers employed the Foreman lime-ethanol procedure, although they introduced numerous improvements, including a correction for the solubility of the calcium salts of glutamic and aspartic acids. The value 22 00 was considered to be reasonably quantitative (accurate within 2 per cent).

Glutamic acid as well as other amino acids have been isolated and determined by chromatographic adsorption methods in which artificial resins were used as ion exchangers and activated carbon, zeolites, clays, titania gel, silica gel, and aluminum oxide as adsorbents, although these procedures have been applied largely to mixtures of pure amino acids. Wieland (39) has given a nearly complete review of the literature on this topic.   In 1942, Wieland (40) found 17 0 per cent of glutamic acid isolated as the hydrochloride from the adsorbate on aluminum oxide of an acid hydrolysate of Hammarsten's casein   Quite recently, the glutamic acid in the hydrolysates of three proteins other than casein was determined by Cannan (41), who isolated glutamic acid hydrochloride from the adsorbate on amberlite IR-4 resin.   Kibrick (42) determined the glutamic acid in the same material by calculation from electrometric titration and gasometric ninhydrin data. It appears that these methods should give results satisfactory for many purposes.

Other procedures employed for the determination of glutamic acid in proteins other than casein include (a) titration with ammonium thiocyanate of the succinic acid obtained by deamination and oxidation of glutamic acid (43) and (b) the isotope dilution method (44).

[13] Commercial casein containing 8.35 per cent moisture, 2.54 per cent ash, and 13.59 per cent nitrogen (13.57, 13 60, 13.59) uncorrected and 15.26 per cent corrected for moisture and ash. A 450.0 gm. lot of this casein (equivalent to 401.0 gm. of ash- and moisture-free product) was suspended in a mixture of 1800 ml. of 8 N hydrochloric acid, 50 ml. of concentrated nitric acid, and 5.0 gm. of $FeCl_3 \cdot 6H_2O$. The suspension

formed to provide additional evidence on this point.   A quantity of casein was hydrolyzed and glutamic acid determined.   All operations were carried out under quantitative conditions with calibrated apparatus.   Two crops of glutamic acid hydrochloride were isolated, and the three filtrates were assayed for glutamic acid by the microbiological procedure described in the present paper.   The total amount of glutamic acid obtained in this manner was 90.4 gm. or 22.5 per cent of the casein calculated on an ash- and moisture-free basis   (Table VII).

There is reason to believe that this value is accurate, because the intrinsic errors are probably of small magnitude.   Only small amounts of $l(+)$-glutamic acid are lost by adsorption on carbon during decolorization (45), by conversion to humin (46) and pyrrolidonecarboxylic acid (47), and by racemization (48, 49) under the observed experimental conditions.   The latter point is particularly significant, since, as shown by the curves in Fig. 2, less acid is produced by *Lactobacillus arabinosus* 17-5 in the presence of *dl*- than of $l(+)$-glutamic acid at the same relatively low concentration of these forms, while more acid is produced by *dl*- than by $l(+)$-glutamic acid at the same relatively high concentration of the two forms.   It may be inferred from these observations that the unnatural antipode of glutamic acid plays some essential rôle in the metabolism of this microorganism.   It is also evident that the microbiological procedure described is inapplicable to solutions containing mixtures of $l(+)$- and *dl*-glutamic acid.

It is considered that the present value, 2.03 per cent, for the yield of glutamic acid from silk fibroin is reasonably accurate, although this assumption cannot be supported by comparison with values in the literature. Fischer (50) isolated 11.7 per cent of glutamic acid from the fibroin prepared from the silk of a species of large spider from Madagascar, but it appears from the literature[14] that only from 0 to 3 per cent of glutamic

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was heated on a boiling water bath for $1\frac{1}{2}$ hours and then refluxed for 8 hours.   The humin (5.8 gm , dry weight) was removed, the solution was decolorized with nuchar XXX, and 2 crops of glutamic acid hydrochloride were isolated, purified, and analyzed.   The three filtrates were assayed for glutamic acid by the microbiological procedure described.   The total weight of decolorizing carbon used was 65.5 gm. and the dry, residual carbon recovered was 75.1 gm   The 9 6 gm. of material adsorbed represented 2.1 per cent of the original casin   The results of this experiment are summarized in Table VII.

[14] According to Abderhalden and other workers the fibroin derived from the silk of silkworms grown in various localities contains the following percentages of glutamic acid: New Chwang 1 7 (51), Niet ngō tsam 3 0 (52), Schantung-Tussah 1.75 (53), Bengal trace (54), Canton 0 (55), Grège Tussah 1 0 (56), *Antheraea pernyi* trace (57), *Antheraea yamamai* 0 6 (57), *Caligula japonica* trace (57), Cheefoo 2.0 (58, 59), and Tai Tsao Tsam 2.0 (45).   The probable accuracy of these values cannot be estimated from the experimental data reported.

acid has been found in the fibroin from the silkworm. It has been empha-
sized by Sah *et al.* (60) that the amino acid content of silk fibroin may vary

TABLE VII

Results of Experiment on Isolation and Assay of Glutamic Acid in Casein Hydrolysate

Fraction	Glutamic acid hydrochloride					Glutamic acid calculated from hydrochloride or assayed
	Quantity isolated	Nitrogen		Chloride		
		Found	Theory	Found	Theory	
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*gm.*
Crop I .	68.0	7.96	7.63	20.0	19.3	54.85
" II	20.4	7.58	7.63	21.5	19.3	16.45
Filtrate 1						1.98
" 2						16.07
" 3						1.04
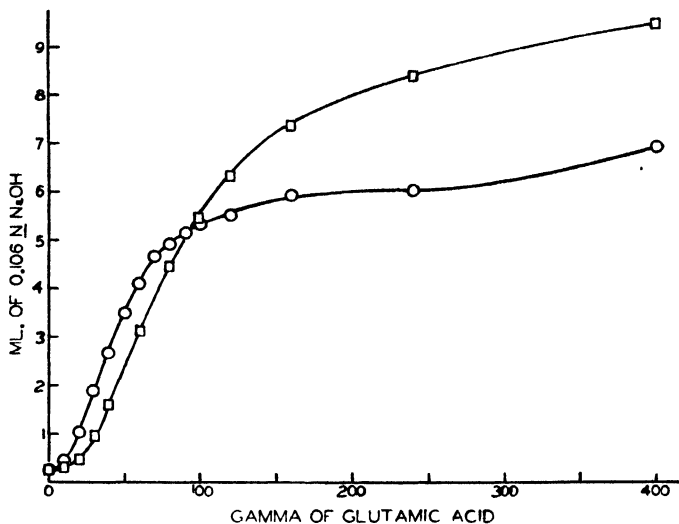
FIG. 2. Curves showing acid production in terms of ml. of standard base at dif-
ferent levels of *l*(+)-glutamic acid (circles) and *dl*-glutamic acid (squares). The
curves were derived from duplicate assays. Maximum variations in the titration
values are denoted by the diameter of the circles and squares. Essentially the
same curves were obtained in three duplicate experiments.

with the locality, climate, and season as well as with the food and species
of the silkworm.

SUMMARY

A method is described for the quantitative determination of *l*(+)-glu-
tamic acid in protein hydrolysates with *Lactobacillus arabinosus* 17-5.

Satisfactorily precise assay data are given which are believed to be accurate. Casein and silk fibroin were found to contain 22.5 and 2.03 per cent of glutamic acid, respectively. The microbiological procedure described is considered to be inapplicable to the assay of $l(+)$-glutamic acid in solutions containing a mixture of $l(+)$- and dl-glutamic acids. It is suggested that the unnatural antipode of glutamic acid may play some essential rôle in the metabolism of the microorganism *Lactobacillus arabinosus* 17-5.

BIBLIOGRAPHY

1. Schott, H. F., Rockland, L. B., and Dunn, M. S., *J. Biol. Chem.*, **154**, 397 (1944).
2. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
3. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
4. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 322 (1944).
5. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
6. Van Slyke, L. L., and Bosworth, A. W., *J Biol. Chem.*, **14**, 203 (1913).
7. Dunn, M. S , Abstracts, American Chemical Society, 107th meeting, Cleveland, 9A (1944).
8. Hutchings, B. L., and Peterson, W H., *Proc Soc Exp. Biol. and Med.*, **52**, 36 (1943).
9. Shankman, S., Dunn, M. S., and Rubin, L. B , *J. Biol. Chem.*, **150**, 477 (1943).
10. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 69 (1932).
11. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 461 (1937).
12. Fischer, E., and Skita, A., *Z. physiol. Chem* , **33**, 177 (1901)
13. Shelton, E. M., and Johnson, T. B., *J. Am. Chem. Soc.*, **47**, 412 (1925).
14. Vignon, M. L., *Compt. rend. Acad.*, **114**, 129 (1892).
15. Vignon, M. L , *Compt rend. Acad* , **113**, 802 (1891); **114**, 603 (1892); **115**, 442, 613 (1892).
16. Harris, M., and Johnson, T. B., *Ind. and Eng. Chem.*, **22**, 965 (1930).
17. Hegsted, D. M., and Wardwell, E. D., *J. Biol. Chem* , **153**, 167 (1944).
18. Foreman, F. W., *Biochem. J.*, **13**, 378 (1919).
19. Vickery, H. B., *J. Biol. Chem* , **132**, 325 (1940).
20. Chibnall, A. C., Bakerian lectures (1942), reprinted from *Proc. Roy. Soc. London*, *Series B*, **131**, 136 (1942).
21. Kuhn, R., Birkofer, L., and Quackenbush, F. W., *Ber. chem. Ges.*, **72**, 407 (1939).
22. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 360 (1943).
23. Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, **9**, 333, 425 (1911).
24. Vickery, H. B., and White, A., *J. Biol. Chem.*, **103**, 413 (1933).
25. Lavine, T. F., *J. Biol. Chem.*, **151**, 281 (1943).
26. Block, R. J., and Bolling, D , The determination of the amino acids, Minneapolis, iii (1942).
27. Dakin, H. D., *Biochem. J.*, **12**, 290 (1918).
28. Nicolet, H. B., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).
29. Shinn, L. A., and Nicolet, H. B., *J. Biol. Chem.*, **138**, 91 (1941).
30. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
31. Hlasiwetz, H., and Habermann, J., *J. prakt. Chem.*, **7**, 397 (1873).

32. Foreman, F. W., *Biochem. J.*, **8**, 463 (1914).
33. Melville, J., reported by Vickery, H. B., *Carnegie Inst. Washington Yearbook*, **35**, 308 (1935–36).
34. Pucher, G. W., and Vickery, H. B., *Ind. and Eng. Chem.*, *Anal. Ed.*, **12**, 27 (1940).
35. Olcott, H. S., *J. Biol. Chem.*, **153**, 71 (1944).
36. Macara, T. J. R., and Plimmer, R. H. A., *Biochem. J.*, **34**, 1431 (1940).
37. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
38. Cohn, E. J., and Hendry, J. L., *J. Gen. Physiol.*, **5**, 521 (1923).
39. Wieland, T., *Chemie*, **56**, 213 (1943).
40. Wieland, T., *Ber. chem. Ges*, **75**, 1001 (1942).
41. Cannan, R. K., *J. Biol Chem.*, **152**, 401 (1944).
42. Kibrick, A C , *J Biol Chem.*, **152**, 411 (1944).
43. Arhimo, A A., and Laine, T., *Suomen Kemistilehti*, **12 B**, 18 (1939); *Chem. Abstr.*, **33**, 6198 (1939).
44. Rittenberg, D., and Foster, G L , *J Biol. Chem.*, **133**, 737 (1940).
45. Cheldelin, V. H., and Williams, R. J , *J Am. Chem. Soc.*, **64**, 1513 (1942).
46. Roxas, M. L., *J. Biol. Chem.*, **27**, 71 (1916).
47. Foreman, F. W., *J. Biochem. J* , **8**, 481 (1914).
48. Arnow, L. E , and Opsahl, J. C , *J Biol. Chem* , **133**, 765 (1940).
49. Johnson, J. M., *J. Biol. Chem.*, **134**, 459 (1940)
50. Fischer, E , *Z. physiol. Chem.*, **53**, 127 (1907).
51. Abderhalden, E , and Rilliet, A , *Z physiol. Chem* , **58**, 337 (1908–09).
52. Abderhalden, E., and Brossa, G A , *Z physiol. Chem* , **62**, 129 (1909).
53. Abderhalden, E , and Brahm, C , *Z physiol Chem.*, **61**, 256 (1909).
54. Abderhalden, E , and Sington, J., *Z. physiol. Chem.*, **61**, 259 (1909).
55. Abderhalden, E., and Behrend, L., *Z. physiol. Chem* , **59**, 236 (1909).
56. Abderhalden, E., and Spack, W , *Z. physiol. Chem.*, **62**, 130 (1909).
57. Suzuki, U., Yoshimura, K , and Inouye, R., *J. Coll Agr Imp. Univ. Tokyo*, **1**, 59 (1909).
58. Abderhalden, E., and Welde, E., *Z physiol. Chem* , **64**, 462 (1910).
59. Abderhalden, E., and Schmid, J., *Z physiol. Chem* , **64**, 460 (1910).
60. Sah, P. T., Ma, T.-S., and Ma, S.-Y , *J Chinese Chem. Soc.*, **1**, No. 1 (1933).
61. Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

PHOTOCHEMICAL DESTRUCTION OF RIBOFLAVIN IN MILK AND LOSSES DURING PROCESSING

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It has been adequately demonstrated that the riboflavin in milk is rapidly destroyed upon exposure to direct sunlight (1, 2). Moreover, increased temperature favors an increased destruction of the vitamin (2, 3). Since it is customary, during commercial processing of milk, to carry out pasteurization by heating the raw milk in a thin film, during which time it may be exposed to light in the plant and therefore subject to considerable riboflavin destruction, the loss of the vitamin during this treatment has been investigated.

Samples of fresh milk, obtained before and after pasteurization, were protected from light and refrigerated until the assays were performed, always within 24 hours of collection. Riboflavin determinations were made by the microbiological method (4), and frequent recovery experiments were made with the use of the pure crystalline vitamin in order to check the accuracy of the technique. Recoveries were always within the range of 94 to 102 per cent. Samples of milk were made alkaline, exposed to light for 24 hours under a 750 watt lamp to destroy all riboflavin, neutralized to pH 6.8, and added separately to the basal medium in identical quantities to those being assayed. The results of these determinations were taken as blanks and were subtracted from the values obtained in the actual assays to give the results reported (Table I).

Ultraviolet irradiation has been shown to cause rapid destruction of riboflavin,[1] some spectral regions being more effective than others. Since irradiation is employed in the preparation of "vitamin D-enriched milk," it was decided to determine how much, if any, of the riboflavin was destroyed in this part of milk processing.

Accordingly, samples of milk were obtained before and after irradiation and assayed for riboflavin as described. The results are shown in Table II.

A determination was also made under the conditions of lighting in the plant to learn whether riboflavin was destroyed during the bottling operation. Samples of milk were taken just before bottling and again just before delivery to the consumer. An average of seven determinations

[1] Ziegler, J. A., unpublished work.

indicated that between 3 and 5 per cent of the vitamin B$_2$ was destroyed in this manner.

TABLE I

Destruction of Vitamin B$_2$ during Pasteurization

Experiment No	Pasteurization*	Riboflavin	Destruction
		γ per ml.	per cent
1	Before	2.07	
	After	1.86	10.1
2	Before	2.05	
	After	1.85	9.7
3	Before	1.97	
	After	1.79	9.1
4	Before	2.10	
	After	1.75	16.6

* All samples were assayed in triplicate

TABLE II

Destruction of Riboflavin during Vitamin D Enrichment

Experiment No	Sample*	Riboflavin	Destruction
		γ per ml	per cent
1	Before	1.86	
	Vitamin D-enriched	1.75	5.1
2	Before	1.85	
	Vitamin D-enriched	1.70	8.3
3	Before	1.92	
	Vitamin D-enriched	1.78	7.2

* All samples were assayed in triplicate.

SUMMARY

The riboflavin in milk was found to be destroyed to the extent of 9 to 16 per cent during pasteurization, from 5 to 8 per cent during vitamin D enrichment by irradiation, and from 3 to 5 per cent during bottling and brief storage preceding delivery.   It has previously been shown (2) that up to 66 per cent of the riboflavin is destroyed if milk is exposed for 2 hours to direct sunlight.

BIBLIOGRAPHY

1. Peterson, W. J., Haig, F. M., and Shaw, A. O., *J. Am. Chem. Soc.*, **66**, 662 (1944).
2. Ziegler, J. A., *J. Am. Chem. Soc.*, **66**, 1039 (1944).
3. Williams, R R , and Cheldelin, V. H., *Science*, **96**, 22 (1942).
4. Snell, E. E , and Strong, F M , *Ind. and Eng Chem., Anal. Ed* , **11**, 346 (1939).

STEROIDS

VI. THE STRUCTURE OF PREGNANEDIOL GLUCURONIDE FROM HUMAN PREGNANCY URINE

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In 1936 Odell and Marrian (1) established that pregnane-3(α),20(α)-diol (IV), the chief urinary transformation product in man of the corpus luteum hormone progesterone, is excreted as a water-soluble, acid-hydrolyzable complex which Venning and Browne (2) later in that year isolated from human pregnancy urine as the sodium salt of a conjugate of the diol with 1 mole of a uronic acid, presumably glucuronic acid. Further elucidation of structure is now provided from a study of the degradation of the natural ester, which proves that the uronic acid is coupled to the 3-hydroxyl group of the steroid moiety.[1]

To ascertain the position of conjugation, it was proposed to label the free hydroxyl group of the pregnanediol uronide by oxidation to the ketone or by acetylation, and then to identify the corresponding pregnanolone or pregnanediol monoacetate arising on cleavage of the oxidized or acetylated uronic acid residue. The oxidative approach proved to be abortive, treatment of the ester with chromic anhydride at room temperature gave a mixture from which no crystalline material could be separated either directly or after acid or alkaline hydrolysis. On acetylation, however, an ether-soluble crystalline polyacetate was obtained in 85 per cent yield. The observed carbon, hydrogen, and acetyl content and the molecular weight clearly indicated the introduction of three acetyl groups and the loss of 1 mole of water. That lactonization also took place under treatment with acetic anhydride was revealed by the analytical values, the insolubility of the substance in cold aqueous alkali, and the formation of a soluble sodium salt on heating. Accordingly the product is assigned the triacetate lactone structure (II).

Conditions were then sought (Table I) which would effect maximum hydrolysis of the glycosidic linkage of (II) with minimum cleavage of the acetyl group marking the free alcoholic function of the steroid. In each experiment, the neutral ether-soluble products of hydrolysis were separated by chromatographic analysis. To serve as a model, a mixture of pregnane-

diol, its diacetate, and two monoacetates was first prepared as described by Hirschmann (4) and fractionated chromatographically. The diacetate, m.p. 180–181°, was eluted with mixtures of benzene and petroleum ether containing up to 20 per cent of benzene; the 3-monoacetate, m.p. 155–159°, with benzene (20 to 60 per cent)-petroleum ether; the 20-monoacetate,
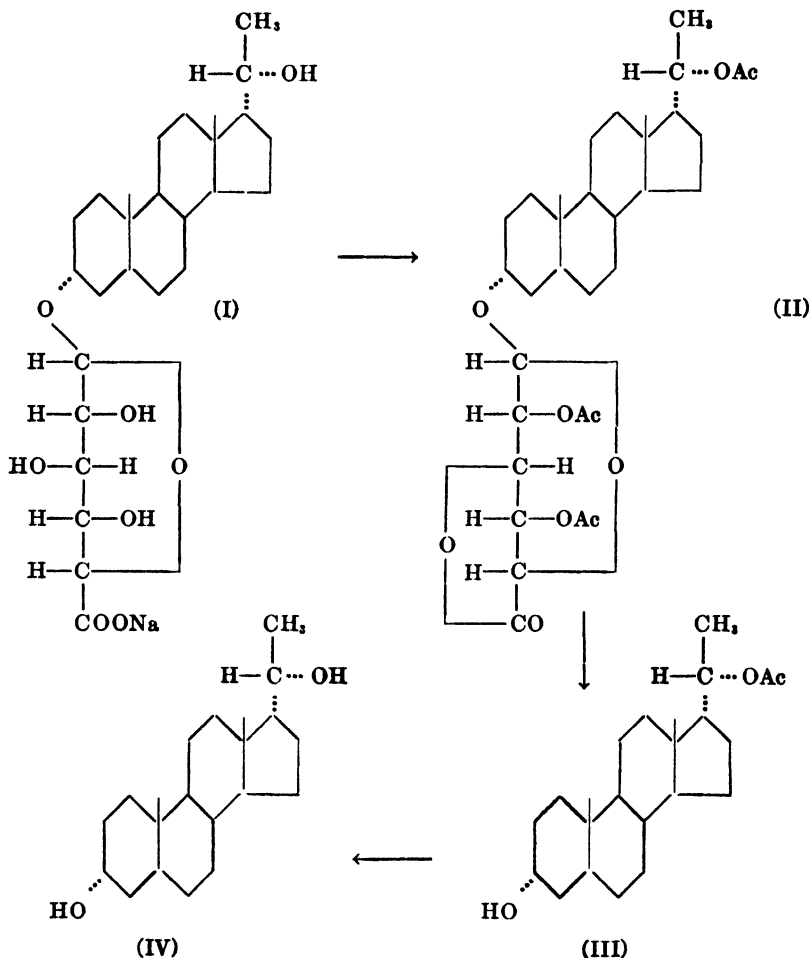
m.p. 173–174°, with benzene (>70 per cent)-petroleum ether, absolute
benzene, and acetone (10 per cent)-benzene; and pregnanediol, m.p. 238–
240°, with acetone (>10 per cent)-benzene. The fractions separated from
the chromatograms of the' hydrolysates of (II) are listed in Table I as
(a) early eluates, eluted with benzene (up to 85 per cent)-petroleum ether,
(b) pregnanediol 20-monoacetate, eluted with benzene (>70 per cent)-
petroleum ether, absolute benzene, and acetone (5 per cent)-benzene,
(c) middle eluates, eluted with acetone (5 to 10 per cent)-benzene, which
comprised mixtures of the 20-monoacetate and pregnanediol, (d) pregnane-
diol, eluted with acetone (10 to 30 per cent)-benzene, and finally (e) late
eluates, separated with acetone (>30 per cent)-benzene. The negligible

TABLE I

Acid Hydrolysis of Acetylated Pregnanediol Glucuronide

Experiment No.	Triacetate lactone	Hydrolysis for 1.5 hrs. in ethanolic HCl	Neutral fraction after hydrolysis								
			Weight before adsorption	Chromatographic separation							
				Early eluates	Pregnanediol 20-monoacetate		Middle eluates	Pregnanediol		Late eluates	Total eluates
	mg	*N*	*mg.*	*mg*	*mg.*	*per cent yield**	*mg.*	*mg.*	*per cent yield**	*mg.*	*mg*
1	150	0.05	68.6	4.3	34.1	38	1.8	13.5	17	·7.4	61.1
2	163	0.05	97.8	2.0	41.4	42	0	17.2	13	11.4	72.0
3	300	0.01	202.8	19.9	24.9	14	0	13.6	8	10.3	68.7
4	600	0.5	301.2	2.4	50.4	14	28.4	184.8	61	4.8	270.8
	Pregnanediol 20-monoacetate										
5	43.6	0.05	37.4	2.5	20.7	55	2.5	10.7	28	1.1	37.8

* Calculated as per cent of theory from the triacetate lactone (Experiments 1
to 4).

quantity of material obtained in the early eluates, which from the model
should contain any 3-monoacetate, could not be induced to crystallize.
Obtained only were pregnanediol 20-monoacetate (III), identified by mix-
ture melting point determination and by oxidation to pregnan-20-ol-3-one
acetate, and a smaller amount of pregnanediol (IV), which arises on further
hydrolysis of (III), as demonstrated by its production from the 20-mono-
acetate by the same acid treatment (Experiment 5, Table I). Optimum
partial hydrolysis of (II) was realized on refluxing 1.5 hours in 0.05 N
ethanolic hydrochloric acid (Experiments 1 and 2) which gave about
40 per cent of the monoacetate (III) and about 15 per cent of the diol (IV).
With stronger acid (Experiment 4), hydrolysis proceeded more nearly to

completion to yield 61 per cent of (IV) and only 14 per cent of (III). Under milder conditions (Experiment 3) hydrolysis was very incomplete (respectively only 14 and 8 per cent of (III) and (IV)) and the bulk of the material (66 per cent) remained uneluted from the alumina, owing to the tenacious adsorptive properties of the unhydrolyzed uronic acid residue.

Thus the absence of the 3-monoacetate and the presence of large amounts of the 20-monoacetate in the hydrolysate of the acetylated natural ester clearly establish the conjugation between the 3-hydroxyl group of pregnanediol and the glucosidic hydroxyl of the uronic acid.

EXPERIMENTAL

Melting points were taken with the Fisher-Johns micro apparatus; the recorded values are uncorrected.

Triacetate Lactone of Pregnanediol Glucuronide (II)—Sodium pregnanediol glucuronide ((I) 1.5 gm.)[2] dissolved in acetic anhydride (150 ml.) with gentle warming was allowed to stand at room temperature for 48 hours, when the solution was diluted to 10 volumes and thrice extracted with ether. The combined ethereal extracts 'were washed with N sodium hydroxide solution (three times) and water (five times) and taken to dryness (1.287 gm.). The melting points of the products of several acetylations ranged, after one crystallization from aqueous ethanol, from 95–110° to 110–117°. For analysis a sample was twice recrystallized (m.p. 123–125°) and dried over P_2O_5 at 65° and 0.1 mm. for 3 hours.

Analyses—$C_{21}H_{34} \cdot C_6H_5O_4$ ($OCOCH_3$)$_3$

Calculated.	C 65 60,	H 8 02,	mol. wt	(Rast) 604,	acetyl value 21 3
Found.	" 65.24,	" 8.34,	" "	" 610,	" " 21.78
"	" 65 16,	" 8 43,	" "	" 631,	" " 22.06

From cold ethanolic potassium hydroxide (10 per cent) solution the product was precipitated on addition of water. After it was refluxed (2 hours) in ethanolic potassium hydroxide (10 per cent) solution, a soluble salt was formed, as evidenced by the failure to obtain a precipitate on dilution.

Separation of Pregnanediol and Acetates of Pregnanediol—A mixture of pregnanediol and its acetates was prepared as described by Hirschmann (4) by refluxing the former (1.967 gm.) for 2 hours in glacial acetic acid. After dilution to 10 volumes, the mixture was thrice extracted with ether and the combined ethereal extracts were washed neutral with 0.1 N sodium hydroxide solution and water. When the neutral residue obtained on distillation of the solvent was leeched with five portions of hot petroleum ether, 360 mg. of unchanged pregnanediol remained undissolved. This

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was treated again with acetic acid, and worked up as before; 102 mg. of crude pregnanediol remained in the petroleum ether-insoluble fraction. Evaporation of the combined petroleum ether leechings gave 1.82 gm. of solid material which was dissolved in benzene, brought to the point of precipitation by the addition of petroleum ether, adsorbed on a column (35 × 1.5 cm.) of alumina (Merck, standardized according to Brockmann, 54 gm.) previously saturated with petroleum ether, and fractionally eluted as outlined in Table II. Fractions 3 to 16 (621.7 mg.) exhibited the characteristic double melting point of slightly impure pregnanediol diacetate;

TABLE II

Chromatographic Separation of Pregnanediol and Its Acetates

Fraction No	Eluent (50 ml portions)		Eluate		Compounds isolated
			Yield	Nature or melting point	
			mg.	*°C.*	
1– 2	Petroleum ether		0.0		
3– 9	Benzene (10%)-petroleum ether		454.4	⎱148–160	Pregnanediol diace-
10–16	" (20%)- " "		167.3	⎰170–177	tate
17–19	" (30%)- " "		1.6	Oil	
20–22	" (40%)- " "		213.3	98–111	⎱
23–25	" (50%)- " "		62.5	98–111	Pregnanediol
26–28	" (55%)- " "		93.8	98–111	3-monoacetate
29	" (60%)- " "		10.1	98–111	⎰
30–31	" (60%)- " "		10.5	Oil	
32	" (70%)- " "		11.0	"	
33	" (70%)- " "		34.0	138–154	⎱
34–38	" (85%)- " "		119.9	161–170	Pregnanediol
39–43	"		82.8	162–169	20-monoacetate
44–45	Acetone (10%)-benzene		36.1	135–160	⎰
46	" (10%)- "		20.4	209–211	⎱
47–49	" (20%)- "		254.7	209–211	Pregnanediol
50	"		39.0	209–211	⎰

two recrystallizations from benzene-petroleum ether resulted in the product melting sharply at 180–182°. The 3-monoacetate (379.7 mg., Fractions 20 to 29), after one recrystallization from aqueous ethanol, melted at 100–111° and 124–127°, and was not further purified; Hirschmann (4) records a melting point of 131.5–132.5° (corrected). Impure pregnanediol 20-monoacetate (272.8 mg.) separated with higher concentrations of benzene (Fractions 33 to 45); a second flowing chromatogram and recrystallization from aqueous ethanol raised the melting point to 175–177°. The later eluates (Fractions 46 to 50) gave up 314.1 mg. of unacetylated pregnanediol in the crude state.

The above partition chromatogram differs from that already described by Hirschmann (4) in that a greater concentration of the more active solvent is required for the elution of each of the components, and proportionally more of the fully acetylated product is obtained.

Hydrolysis of Triacetate Lactone of Pregnanediol Glucuronide—Of the several hydrolyses carried out under the different conditions set out in Table I, Experiment 1 only is described in detail.

Crude substance (II) (150 mg.) in 0.05 N ethanolic (70 per cent) hydrochloric acid solution (20 ml.) was refluxed 1.5 hours. The reaction mixture was chilled, neutralized to pH 6, freed of most of the ethanol by distillation *in vacuo*, diluted to 10 volumes, and extracted with ether (four times with 40 ml.). After the material was washed with 10 per cent sodium carbonate solution (three times with 30 ml.) and water (four times with 30 ml.), the combined ethereal extracts were dried (sodium sulfate) and evaporated (68.6 mg. of a solid neutral fraction). Acidification of the carbonate washings, collection of the product with ether, and removal of the solvent yielded 24.2 mg. of acids which were not further investigated. The original aqueous phase after extraction with ether exhibited a strongly positive naphthoresorcinol test for uronic acid and reduced Benedict's reagent.

The neutral hydrolytic products (68.6 mg.), dissolved in benzene and brought almost to the point of precipitation by the addition of petroleum ether, were adsorbed on a column (9.5 × 1.0 cm.) of alumina (2.0 gm.; E. Merck, standardized according to Brockmann), which had previously been saturated with petroleum ether, and fractionally eluted (Table III). The early eluates (Fractions 5 to 20) failed to give up pregnanediol 3-monoacetate on attempted crystallization. Fractions 21 to 34 yielded 34.1 mg. of the 20-monoacetate (III) (m.p. 167–172°), which, after one recrystallization from aqueous ethanol, had a melting point of 173–175° and showed no depression on admixture with authentic 20-monoacetate (m.p. 174–176°) prepared by partial acetylation of pregnanediol.

For further identification, 10 mg. were oxidized (16 hours at room temperature) in 1 ml. of 90 per cent acetic acid containing 14.6 mg. of chromic anhydride. The oxidation product was collected with ether and washed free of acids in the usual way. The recrystallizations of the neutral residue from aqueous ethanol gave pregnan-20-ol-3-one acetate (2.6 mg.) melting at 140–144°; Butenandt *et al.* (5) and Marker *et al.* (6) record a melting point of 144–145° (uncorrected).

Fractions 37 to 41 (13.5 mg., m.p. 233–236°) gave on recrystallization from aqueous ethanol pure pregnanediol, which melted at 238–240° and showed no depression of melting point on admixture with the authentic diol (m.p. 238–240°).

TABLE III

Chromatographic Separation of Products of Hydrolysis of Acetylated Pregnanediol Glucuronide

Fraction No.	Eluent (10 ml portions)	Eluate		Compounds isolated
		Yield	Nature or melting point	
		mg.	*°C.*	
1- 4	Petroleum ether	2.5	Oil	
5- 6	Benzene (20%)-petroleum ether	0.9	"	⎫
7- 8	" (30%)- " "	0.4	"	⎪
9-10	" (40%)- " "	0.0		⎪
11-12	" (50%)- " "	0.5	Oil	Early eluates failed
13-14	" (60%)- " "	0.0		to crystallize
15-16	" (70%)- " "	0.0		⎪
17-20	" (85%)- " "	0.0		⎭
21	" (85%)- " "	1.0	169-171	⎫
22-30	"	27.5	168-172	Pregnanediol 20-
31-34	Acetone (5%)-benzene	5 6	167-171	monoacetate
35-36	" (10%)- "	1.8	169-180	Middle eluates
37-41	" (20%)- "	13.5	233-236	Pregnanediol
42-43	" (30%)- "	0.0		
44-45	" (75%)- "	1.0	Oil	⎫
46-47	"	4.4	"	Late eluates
48-49	Ethanol	2.0	"	⎭
Total .. .		61.1		

SUMMARY

Acetylation of the sodium salt of pregnanediol glucuronide from human pregnancy urine yields a crystalline non-acidic polyacetate, the analyses and properties of which indicate a triacetate lactone. Hydrolysis of the glucosidic linkage of the latter sets free the 20-monoacetate of pregnanediol. Thus it is established that, in the natural ester, the glucuronic acid is conjugated with the 3-hydroxyl group of the steroid moiety.

BIBLIOGRAPHY

1. Odell, A. D., and Marrian, G. F., *Biochem. J.*, **30**, 1533 (1936).
2. Venning, E. M., and Browne, J. S. L., *Proc. Soc. Exp. Biol. and Med.*, **34**, 792 (1936).
3. Huebner, C. F., Overman, R. S., and Link, K. P., *J. Biol. Chem.*, **155**, 615 (1944).
4. Hirschmann, H., *J. Biol. Chem.*, **140**, 797 (1941).
5. Butenandt, A., Westphal, U., and Hohlweg, W., *Z. physiol. Chem.*, **227**, 84 (1934).
   Butenandt, A., and Schmidt, J., *Ber. chem. Ges.*, **67**, 1893 (1934). ·
6. Marker, R. E., Kamm, O., Wittle, E. L., Oakwood, T. S., Lawson, E. J., and Laucius, J. F., *J. Am. Chem. Soc.*, **59**, 2291 (1937).

THE SYNTHESIS OF PREGNANEDIOL 3-β-d-GLUCURONIDE*

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The work of Marrian and coworkers (1, 2) and Venning and Browne (3) has shown that certain sex hormones occur in the urine as conjugated uronides. A conjugate of pregnanediol was isolated by Venning and Browne, the uronic acid presumably being glucuronic. By the identification of pregnanediol 20-acetate among the hydrolysis products of a pregnanediol uronide "polyacetate" derived from the natural conjugate, Heard and coworkers have recently shown that the carbohydrate residue is linked to the 3-hydroxyl group of the steroid (4).

In this paper we report the synthesis of pregnanediol 3-β-d-glucuronide, a compound which is identical with the natural pregnanediol glucuronide of Venning and Browne (3). Thus by an independent method it is shown that the hydroxyl group in position 3 of the pregnanediol moiety is linked with carbon atom 1 of the d-glucuronic acid (pyrano ring). Veitch et al. (5) indicated that the pregnanediol glucuronide isolated from urine was hydrolyzed by emulsin. This fact and the method of synthesis used by us permit assignment of the β configuration to the glucuronide with some confidence.

Pregnanediol 20-acetate (6) was coupled with acetobromo-d-glucuronic acid methyl ester (7) in benzene in the presence of silver oxide to yield pregnanediol 20-acetyl-3-(triacetyl-β-d-glucuronide methyl ester) which on treatment with methanolic sodium hydroxide gave sodium pregnanediol 3-β-d-glucuronidate. The former compound was also realized by methylation and acetylation of the natural pregnanediol glucuronide. The physical constants of the synthetic and natural product are in agreement.

The occurrence in nature and the synthesis of pregnanediol glucuronide (the steroid portion of which has an *epi* configuration) sustain the work of Linstead (8), which refutes the contention of Miescher and Fischer (9) that episteroids do not form glycosides.

Steroid galacturonides have been synthesized by Sell and Link (10) and Shapiro (11) has prepared certain glucuronides in the sex hormone class.

For control purposes in this work, we prepared stigmasterol β-d-glucuronide as the triacetyl methyl ester and pregnanediol 3-β-d-glucoside as the tetraacetate.

Preparation of Pregnanediol 20-Acetyl-3-(triacetyl-β-d-glucuronide Methyl Ester) and Its Conversion to Pregnanediol 3-β-d-Glucuronide—A benzene solution (25 ml.) of 1.00 gm. of pregnanediol 20-acetate and 1.33 gm. of acetobromo-d-glucuronic acid methyl ester was shaken with 2.0 gm. of silver oxide. Coupling was complete in 24 hours. The silver salts were centrifuged off, washed with benzene, and the benzene solution concentrated *in vacuo.* The resulting syrup was dissolved in 10 ml. of hot ethanol and the product was recrystallized from ethanol five times; yield 0.54 gm., m.p. 191–192°.

Analysis—$C_{36}H_{54}O_{12}$ Calculated.   C 63.7, H 8.0, OCH_3 4.6
                         Found.   " 63.2, " 8.0,   "   4.5
Rotation—$[\alpha]_D^{20}$ = +7.5° in benzene, c = 1 per cent

This product was converted to sodium pregnanediol 3-β-d-glucuronidate by refluxing 100 mg. for 3 hours in 15 ml. of 0.5 N sodium hydroxide in methanol. The solution was concentrated *in vacuo.* The crystals that separated during the concentration were filtered and washed with a small volume of cold methanol, followed by a small volume of cold water. After recrystallization from methanol, 30 mg. of the sodium pregnanediol glucuronidate, m.p. 274–275°, were realized. The melting point of a mixture of the natural and synthetic products was 274–275°. The synthetic sodium salt was converted to its acid, pregnanediol 3-β-d-glucuronide, as directed by Venning and Browne (3), m.p. 178–180°. A mixture of the natural and synthetic pregnanediol glucuronides melted at 178–180°.

Preparation of Pregnanediol 20-Acetyl-3-(triacetyl-β-d-glucuronide Methyl Ester) from Natural Pregnanediol Glucuronide—An excess of diazomethane was distilled into a solution of 0.50 gm. of the natural pregnanediol glucuronide in 20 ml. of ethanol. After standing for 2 hours, the ethanol was removed *in vacuo* and the resulting syrup refluxed with 0.5 gm. of sodium acetate in 10 ml. of acetic anhydride for 30 minutes. The mixture was poured into ice water and the acetate purified in the usual way. After five recrystallizations from ethanol the yield was 150 mg., m.p. 188–189°. The melting point of a mixture of the synthetic pregnanediol 20-acetyl-3-(triacetyl-β-d-glucuronide methyl ester) and the corresponding product from the natural source was 188–191°; $[\alpha]_D^{20}$ = +8.0° in benzene, c = 1 per cent.

Preparation of Pregnanediol 20-Acetyl-3-(tetraacetyl-β-d-glucoside)—Pregnanediol 20-acetate (1.00 gm.) and aceto-d-bromoglucose (1.30 gm.) were dissolved in 25 ml. of benzene and shaken with 2 gm. of silver oxide for

24 hours. The reaction mixture was worked up in the usual manner. The product was recrystallized three times from ethanol; yield 0.36 gm., m.p. 200–204°.

Analysis—$C_{37}H_{56}O_{12}$. Calculated, C 64.1, H 8.1; found, C 63.9, H 7.8
Rotation—$[\alpha]_D^{20} = +6.1°$ in benzene, $c = 1.5$ per cent

Preparation of Stigmasterol Triacetyl-β-d-glucuronide Methyl Ester—0.5 gm. of stigmasterol and 0.5 gm. of acetobromo-*d*-glucuronic acid methyl ester were dissolved in 10 ml. of benzene and shaken with 1 gm. of silver oxide for 24 hours. The benzene extract from the silver salts was concentrated to a syrup which was dissolved in a small volume of hot acetone. On cooling to room temperature some unchanged stigmasterol separated. The filtrate was concentrated to dryness and the residue after two recrystallizations from ethanol yielded 0.24 gm. of the desired product; m.p. 172–174°.

Analysis—$C_{42}H_{64}O_{10}$. Calculated. C 69.2, H 8.8, OCH_3 4.3
                  Found.   " 69.3, " 8.6, "   4.2
Rotation—$[\alpha]_D^{21} = -52°$ in benzene, $c = 1.9$ per cent

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SUMMARY

1. The naturally occurring uronic acid conjugate of pregnanediol has been shown by synthesis to be pregnanediol 3-β-*d*-glucuronide.

2. The synthesis of pregnanediol 20-acetyl-3-(tetraacetyl-β-*d*-glucoside) and stigmasterol triacetyl-β-*d*-glucuronide methyl ester is also given.

BIBLIOGRAPHY

1. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, **30**, 57, 2250 (1936).
2. O'Dell, A. D., and Marrian, G. F., *Biochem. J.*, **30**, 1533 (1936).
3. Venning, E. M., and Browne, J. S. L., *Proc. Soc. Exp. Biol. and Med.*, **34**, 792 (1936).
4. Heard, R. D. H., Hoffman, M. M., and Mack, G. E., *J. Biol. Chem.*, **155**, 607 (1944).
5. Veitch, F. P., Milone, H. S., and Everitt, E. L., *Science*, **88**, 551 (1938).
6. Butenandt, A., and Schmidt, J., *Ber. chem. Ges.*, **67**, 1893 (1934).
7. Goebel, W. F., and Babers, F. H., *J. Biol. Chem.*, **106**, 63 (1934).
8. Linstead, R. P., *J. Am. Chem. Soc.*, **62**, 1766 (1940).
9. Miescher, K., and Fischer, W., *Helv. chim. acta*, **21**, 336 (1938).
10. Sell, H. M., and Link, K. P., *J. Biol. Chem.*, **125**, 235 (1938).
11. Shapiro, E., *Biochem. J.*, **33**, 385 (1939).

NUCLEIC ACIDS OF RAT LIVER AND HEPATOMA: THEIR METABOLIC TURNOVER IN RELATION TO GROWTH*

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It is well known that animal and plant tissues in general contain two types of nucleic acid associated with basic proteins: (1) thymonucleic acid (desoxyribonucleic acid) occurring in the nucleus, and (2) ribonucleic acid (*d*-ribonucleic acid) found in the cytoplasm and in the nucleolus (1).

The results of recent work suggest certain contrasts between the functions of these closely related substances. Thymonucleic acid has a high capacity for polymerization in long chains. It has been noted that the nucleotide units are spaced at the same distance as are the units in polypeptide chains (2). Its presence in highly polymerized form in the dividing cell is thought to facilitate the orderly splitting of chromosomes (3). It has been thought that thymonucleic acid is attached to chromosome proteins at metaphase, but it has recently been suggested (4) that it pervades the entire nuclear sap and that its gelation is responsible for the mitotic spindle. Although certain aspects of the question are controversial at present, both views appear to agree in giving thymonucleic acid a primarily structural rôle in the nucleus and in suggesting that its physical properties are important for cell division among higher organisms.

Ribonucleic acid is more universally present in life than is thymonucleic acid, as the latter is thought to be absent from most viruses. It is found (5) in cytoplasmic granules and it has been pointed out (6) that its concentration is highest in those tissues in which there is active protein synthesis. It is frequently and perhaps invariably present in nuclei, although as a small part of the nuclear nucleic acid. There is some evidence that nuclear thymonucleic acid is formed from cytoplasmic ribonucleotides, since radiation of tumors results in accumulation of the latter at the expense of synthesis of the former (7). These facts suggest that ribonucleic acid may have a more active or dynamic part in tissue metabolism.

In a preliminary paper (8) we demonstrated that the rate of turnover of thymonucleic acid with respect to inorganic phosphate is dependent on the growth rate of the tissue. With radioactive phosphorus as a tracer, a small but appreciable turnover of this fraction was found to occur in normal

resting liver, whereas in regenerating liver a great increase in the uptake of phosphorus by thymonucleic acid took place and could be correlated with the known growth rate. A different rate of turnover of ribonucleic acid was suggested by the relatively higher rate of phosphorus turnover in the total protein fraction containing both nucleic acids.

The new understanding of cytology and nucleic acid metabolism (9, 10) has naturally added weight to the old view that nucleic acids may be important in regulation of growth; it has indeed been suggested (11) that certain peculiarities of malignant tumors depend on specific changes in the nucleic acid aggregations in chromatin. It therefore seemed desirable to isolate the two nucleic acids in pure form from resting and regenerating liver and hepatoma and to study their phosphorus turnover.

Plan of Experiments

In these experiments, minimal tracer amounts of radioactive phosphorus (P^{32}) have been administered parenterally to rats in the form of inorganic phosphate. Specimens of liver were later removed; nucleic acids and other chemical fractions of whole tissue and of isolated nuclei were then obtained and analyses made for total and radioactive phosphorus. The results have been expressed as specific activity, which is the ratio of radioactive to total phosphorus (millimicrocuries of P^{32} per mg. of P).

Mathematical analyses of specific activity changes in experiments of this type have been made elsewhere[1] (12) and their meaning discussed (13). Briefly, injected labeled phosphate will distribute itself throughout the extracellular phosphate of the body, so that specific activity of extracellular phosphate will tend to reach a level. Through transfer of phosphate into cells, the labeled phosphorus will become further diluted, thus decreasing specific activity in the extracellular compartment.

Owing to turnover of phosphorus between inorganic and organic forms, labeled phosphorus will then appear in the various organic compounds, in exchange for their (initially) unlabeled phosphorus. Obviously, the rate of turnover occurring between any two compounds will be reflected in the rate at which their specific activities, if originally different, approach equality.

In the case of thymonucleic acid, its "immediate precursor" (12), i.e. the immediate source of its phosphorus, is unknown, and it is possible that a phosphorylation cycle may exist. Observation of the rate of exchange of its phosphorus with that of known labile compounds does not, therefore, give evidence as to the mechanism of this exchange. The stability of its phosphorus with respect to inorganic phosphate, as observed in preliminary

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[1] Cohn, W. E., and Brues, A M , data in preparation for publication.

work (8), might therefore be due either (a) to a very slow rate of exchange directly with a precursor among the known labile forms, or (b) to the existence of an unknown series of intermediates having the net effect of retarding its formation from labile phosphorus.

The first experiment was designed to observe the rate at which specific activity of organic phosphorus compounds increased after injection of labeled inorganic phosphate. To avoid too high a specific activity of initial inorganic phosphate, or too great fluctuations in this value, the tracer was given in divided doses throughout the experiment.

In another experiment advantage was taken of the fact that new thymonucleic acid appears to be rapidly synthesized out of more labile substances, during the period of rapid growth (8). Thus it was possible by means of a single P^{32} injection at the height of liver regeneration to build new thymonucleic acid of high specific activity. Following this, when growth again became essentially stationary, spècific activity of inorganic phosphate was brought to a low level by replacing labeled phosphate with unlabeled dietary phosphate. This was done with the aid of parathormone and high phosphate intake. The stability of organic phosphorus compounds would then be reflected at the rate at which their specific activities tended to reach equilibrium with that of inorganic phosphate, in this case in the reverse direction.

By the two experiments it is possible to observe both components of turnover (synthesis and breakdown). Assuming a steady state with no net synthesis or breakdown, the two processes should occur at equal rates.

In a further experiment, we observed the turnover of nucleic acids in a tumor of liver cell origin in a search for a possible specific anomaly of tumor nucleic acid metabolism. In this case, a single initial P^{32} injection was made and the tumors were removed for analysis 3 days later.

EXPERIMENTAL

Sixty-eight female rats of the Sherman strain, 6 to 9 months old, weighing between 180 and 250 gm., were employed in the experiments on normal and regenerating liver, divided into seven groups. Within each group the rats were given identical treatment and their livers were pooled. Partial hepatectomies were performed according to the method of Higgins and Anderson (14). In the tumor experiments, three rats, Mendel-Osborne strain, bearing bilaterally transplanted hepatomas were used. The tumor used was Hepatoma 31 obtained from the National Cancer Institute. Radioactive phosphorus (P^{32}) was injected subcutaneously as neutral sodium phosphate in single or divided doses totaling 28 to 40 microcuries per rat. All tissues to be analyzed were removed as quickly as possible after the animals were sacrificed and placed in ice-cold physiological saline; they were then blotted

gently with filter paper and weighed. Care was taken to remove all visible necrotic tissue from tumors before extraction.

5 volumes of ice-cold distilled water were added to the weighed tissues and the mixture homogenized in a Waring blendor. An aliquot was precipitated by adding 40 per cent trichloroacetic acid to a final concentration of 4.7 per cent. The precipitate, after separation of phospholipids, has been termed "total protein." Inorganic phosphate was precipitated from the supernatant by an ammoniacal solution of magnesium and ammonium nitrates. From the remainder of the aqueous extract, thymonucleic and ribonucleic acids were isolated as described under "Methods." Separation of nuclei is also described below.

Removal of lipids from all protein samples was effected by successive 24 hour extractions with 1:2 alcohol-ether, hot alcohol, and 1:2 alcohol-ether. The protein samples were then dried after being washed with ether. All samples were wet ashed with HNO_3 plus a drop of saturated $MgNO_3$ and dry ashed in a furnace (475–500°) overnight. The ashed samples were transferred quantitatively to porcelain capsules and P^{32} determined on a Lauritsen electroscope with a radium standard. Phosphorus was determined on aliquots by the colorimetric method of Fiske and Subbarow (15). Final results have been expressed as specific activity (millimicrocuries of P^{32} per mg. of P).

In Group 1 (resting liver), in order to avoid too high an initial blood concentration of P^{32}, five injections of 3.6 microcuries were given each rat in the first 24 hours, two of 2.9 microcuries in the next 24 hours, and daily injections of 2.3 microcuries thereafter. In Group 2 (regenerating liver) a single injection of 36 microcuries was given 24 hours after partial hepatectomy; 52 hours after injection those which were to survive 2 weeks received 0.5 per cent neutral sodium phosphate as drinking water and were given daily subcutaneous injections of 5 units of parathormone to promote excretion of phosphorus (16). These rats were intended to maintain a high specific activity only during the period of rapid thymonucleic acid synthesis, since it was shown in a previous study (17) that the original hepatic cell number was three-fourths restored between 24 and 96 hours after operation.

Methods

Since no methods were available for the separation of pure thymo- and ribonucleic acids from the same sample of liver, the following procedure was adapted from various published sources. In this procedure, preliminary separation of nucleoproteins from an aqueous extract of tissue is followed by isolation and purification of the nucleic acids.

Huiskamp (18) obtained a good separation of the two nucleoproteins by precipitation of thymonucleohistone with calcium chloride (0.1 per cent), but the separation appears unsatisfactory in the case of liver, and so we

have precipitated both nucleoproteins at 0.4 per cent calcium chloride. The thymonucleoprotein is first separated from the precipitate with either 10 per cent or 1 M NaCl (19–21); then ribonucleic acid is removed from the protein residue by boiling 10 per cent NaCl, which according to Jorpes (22) does not alter the composition of the acid. Alkaline hydrolysis, as described by Levene and Bass (23), cannot be used to obtain thymonucleic acid without at the same time hydrolyzing ribonucleic acid, owing to the instability of the latter in alkaline solutions (24–27), but we have employed alkali in the final purification of thymonucleic acid to remove contaminating ribonucleic acid. It has finally been purified by Hammarsten's procedure (20). Ribonucleic acid has been purified by precipitation of the barium salt (22), then by precipitation of the free acid in glacial acetic acid (28).

Preliminary Separation—Pooled rat livers and hepatomas (50 to 90 gm.) were ground to a fine brei in a Waring blendor with 5 volumes of ice-cold distilled water and allowed to extract for 2 hours at 0–4° with continuous stirring. A fine white precipitate formed which settled slowly. The calcium-precipitated nucleoproteins were centrifuged and washed once quickly with cold water.

Thymonucleic Acid—From the calcium precipitate, thymonucleoprotein was extracted with about 200 ml. of cold 1 M NaCl for 12 hours. The mixture was then centrifuged at 3000 R.P.M. for 1 hour (high speed centrifugation, as used by Mirsky and Pollister (19), would probably facilitate separation) and the opalescent supernatant siphoned off; the extraction was repeated. The solution, on saturation with NaCl, became viscous and a protein precipitate formed. In later preparations, we treated the calcium precipitate directly with saturated NaCl. After at least 24 hours the combined saturated NaCl solutions were centrifuged and the supernatant poured into 2 volumes of 95 per cent alcohol. The clear alcoholic solution was decanted and the remainder centrifuged. The precipitate was dissolved in water, the insoluble protein residue discarded, and the supernatant poured into 2 volumes of alcohol. This precipitate was white and stringy and could be wound up on a wooden stick; its water solution was clear and highly viscous. Thymonucleic acid was precipitated by addition of 10 per cent HCl, and the sticky precipitate was dissolved in 25 ml. of water with the addition of an excess of 5 N NaOH; the strongly alkaline solution was left 2 hours at room temperature to hydrolyze ribonucleic acid. After neutralization, the free thymonucleic acid was obtained by Hammarsten's procedure (20). The neutralized solution (about 30 ml.) was poured with stirring into 100 ml. of N/3 HCl and the mixture poured at once into 200 ml. of N/3 HCl in 95 per cent alcohol. The resulting fine precipitate was washed with 70 and 95 per cent alcohol and ether and dried at 90° for 1 hour.

Ribonucleic Acid—The calcium precipitate after thorough extraction with

1 M or saturated NaCl was washed once with water and suspended in about 200 ml. of 10 per cent NaCl. The mixture was boiled for 15 minutes with constant stirring. On centrifugation a clear supernatant was obtained and the 'residue was reextracted in the same way. The combined supernatants were poured into 2 volumes of 95 per cent alcohol and a fine white flocculent precipitate formed at once. The solution was centrifuged and the ribonucleic acid precipitate dissolved in water to give a clear solution. On addition of $\frac{1}{4}$ volume of 20 per cent barium acetate (pH 6.8) a flocculent precipitate formed which was washed immediately with 5 per cent barium acetate and then with 70 per cent alcohol. The barium salt was suspended in 25 ml. of ice-cold distilled water and the tube immersed in ice water. 5 N NaOH was added dropwise with stirring until the solution was clear, when it was at once poured into 200 ml. of glacial acetic acid. The white flocculent precipitate was centrifuged, washed with 70 and 95 per cent alcohol and ether, and dried.

Analyses—The isolated nucleic acids were dissolved in water with sufficient added alkali for solution and analyses were made on aliquots. Phosphorus was determined on ashed samples by the method of Fiske and Subbarow (15). Nitrogen analyses were carried out by micro distillation with selenium oxychloride and hydrogen peroxide as oxidants. Thymonucleic acid was determined by the diphenylamine method with a photoelectric colorimeter (29), being read against standards of known concentration.

Separation of Nuclei—Marshak's method was used (30) with minor modifications. Pooled livers were minced with scissors and then passed through an embryo extractor. The pulp was suspended in 5 volumes of ice-cold 5 per cent citric acid and stirred mechanically for 1 hour. The resulting suspension was very fine and when filtered through two layers of gauze gave very little residue. After low speed centrifugation to remove large tissue particles, an aliquot of the suspension was precipitated by trichloroacetic acid for analyses, and the nuclei were separated from the remainder by repeated centrifugation. The final product was examined microscopically to determine the absence of other tissue elements; about 2 ml. of packed nuclei were obtained from 70 gm. of liver. In extracting nuclei from hepatoma we followed the same procedure except that, because of the scirrhous nature of the tumor, the first citric acid extraction was carried out in the Waring blendor.

Packed nuclei were suspended in a few ml. of water and divided into two equal fractions. One was precipitated by adding trichloroacetic acid and freed of phospholipids (nuclear total protein). Nucleic acid was separated from the remainder of the nuclear suspension; when the suspension was diluted with water, a precipitate settled out which was reversibly soluble on neutralization with N NaOH, forming a clear gel. NaCl was added to

the neutralized preparation to a concentration of 10 per cent and it was heated in a boiling water bath for 15 minutes with stirring. After centrifuging, the precipitate was reextracted with 10 per cent NaCl. This precipitate was freed of phospholipids and dried (nuclear protein residue).

To obtain nucleic acid, the combined supernatants were poured into 2 volumes of 95 per cent alcohol; the resulting precipitate was dissolved in water with addition of a few drops of N NaOH. After centrifugation a small protein residue was discarded; the clear viscous supernatant, which gave a strongly positive Dische test (31) for thymonucleic acid, was neutralized and poured into 4 volumes of alcohol with enough NaCl added to cause flocculation. The precipitate, after centrifugation, was washed thoroughly with 70 and 95 per cent alcohol and ether and dried (nuclear nucleic acid).

Yields of Nucleic Acid from Liver—The following figures represent average values from two experiments involving 50 gm. batches of liver.

When the aqueous extract was precipitated by trichloroacetic acid and the precipitate freed of phospholipids, this total protein preparation weighed 153 mg. per gm. of wet liver and contained 0.56 per cent phosphorus. This protein should contain all of the tissue nucleic acid and has commonly been referred to as the "nucleoprotein" fraction.

When the aqueous extract was precipitated by 0.4 per cent $CaCl_2$, one-half to two-thirds of the protein came down and contained 93.1 per cent of the original phosphorus. Of this somewhat more concentrated nucleo-protein, extraction with cold 10 per cent NaCl (thymonucleic acid separation) removed 27.2 per cent of the original protein phosphorus, and boiling 10 per cent NaCl (ribonucleic acid separation) removed 47.3 more, leaving in the residue after these extractions 18.5 per cent of the original protein phosphorus, which was discarded. In the final purified preparations, 8.6 per cent of the original protein phosphorus was represented in thymonucleic acid and 37.2 per cent in ribonucleic acid. Thus 45.6 per cent of the protein phosphorus (in some experiments more than 50 per cent) appeared in the final purified acids. The ratio of ribonucleic acid to thymonucleic acid phosphorus has been reported in the case of lamb liver as 3.5:1 (32).

The procedure here outlined appears applicable to other tissues; we have been able to isolate both nucleic acids from dog liver, intestine, and kidney, in addition to ribonucleic acid from dog pancreas and thymonucleic acid from dog spleen.

Results

Analyses of the nucleic acid preparations are given in Table I. It is worth noting that the N:P ratios of the thymonucleic acid preparations approximate the theoretical value (1.70), and that our analysis of thy-

monucleic acid from hepatoma shows a normal nitrogen content and N:P ratio, in contrast to the very low nitrogen values which have been reported for certain thymonucleic acid preparations from malignant tumors (33).

N:P ratios in ribonucleic acid preparations are higher in both liver and hepatoma. Although the ratio for a tetranucleotide ribonucleic acid (yeast nucleic acid) is also calculated as 1.70, purified pancreas nucleic acid was shown by Levene and Jorpes (28) to give a higher value for guanine than yeast nucleic acid, falling between the values for a pentanucleotide and a

TABLE I

Analyses of Nucleic Acids from Liver and Hepatoma

Sample		Tissue	Sample	P	N	N P ratio	Nucleic acid in sample*	Thymo-nucleic acid in nucleic acid†
		gm	*mg*	*per cent*	*per cent*		*per cent*	*per cent*
Liver	Ribonucleic Acid 1	68.5	233.9	7.59	14.1	1.86	79.9	7.35
	" " 3	74.7	303.5	7.35	14 2	1.93	77 3	2.16
	" " 5	56 0	259.5	7 18	14 0	1 95	75 6	5 57
	Thymonucleic Acid 1	68 5	41 7	9 02	15 3	1 69	91.3	101.0
	" " 3	74.7	78 3	9.19	15 4	1.68	92.9	100.0
	" " 5	56 0	27 2	8 65	14.5	1.68	87 6	99.1
	Ribonucleic Acid 6‡	51 0	161.2	7 87	14.8	1.88	82 8	1.23
	Thymonucleic Acid 6	51.0	42 5	9 12	15 5	1 70	92 3	101.5
Hepatoma 31	Ribonucleic acid	90 0	218 0	7 10	14 9	2 10	74.5	6.52
	Thymonucleic acid	90 0	55 9	9 04	16 0	1 77	91 5	110.0

* Calculated from the P content, assuming the true values to be 9.54 per cent for ribonucleic acid and 9.89 per cent for thymonucleic acid

† Calculated by dividing the mg of thymonucleic acid determined by the Dische method by total nucleic acid content derived from P analysis

‡ This and succeeding samples were obtained by the simplified method; *i e*, the calcium precipitate was treated directly with saturated NaCl.

hexanucleotide with an additional 1 or 2 moles of guanylic acid; calculation of N:P ratios from their data show correspondingly high values (1.86 to 1.88). Although we have not made guanine analyses, it is clear that the liver and hepatoma nucleic acids resemble pancreas ribonucleic acid with respect to their high N:P ratios.

Turnover. Resting Liver—This group comprises four sets of ten rats (weights 201 to 225 gm.). Table II shows the level of urinary phosphate specific activity from a rat in this series, given identical treatment but kept in a metabolism cage. Specific activities of the phosphorus in various fractions of these livers and of their nuclei appear in Table III, Experiment I.

Thymonucleic acid phosphorus at 72 hours has reached a specific activity

11 per cent of that of the inorganic phosphate; at 192 hours it remains essentially the same, being at this time 20 per cent of the (declining) inorganic phosphate value (Table IV). Ribonucleic acid at 72 hours has reached a specific activity 55 per cent that of the inorganic fraction, and throughout the experiments it will be seen that this is reflected in the comparable values for total protein phosphorus, which contains mainly ribonucleic acid (32). Phospholipid turnover is much higher, as is well known.

The total nuclear protein has a specific activity a little higher than that of tissue thymonucleic acid, although at 72 hours this is only 15 per cent

TABLE II

Specific Activity (Millimicrocuries per Mg.) of Urinary Phosphate over 24 Hour Periods

Days after injection	Specific activities*	
	Experiment I	Experiment II
1	251	316
2	162	164
3	91	123
4– 5	89	92
6– 7	67	50
8	65	42
9–10		29
11–12		26
13–14		21
15–16		21
Total P excreted, *mg.*	43 6	196.6
" P³² " *millimicro-curies*	4980	6530

* Experiment I, resting liver, successive injections, total P³² injected, 40 microcuries. Experiment II, regenerating liver, single initial injection; high phosphate intake and parathormone started on 4th day, total P³² injected, 36 microcuries.

of the inorganic value. This might be explained by the presence of a little ribonucleic acid in nuclei, as has been demonstrated.

It is of interest that in the entire series of experiments, as shown in Table III, specific activities of nuclear phospholipids equal those of total tissue phospholipids. Although it is possible that cytoplasmic phospholipids might be adsorbed on nuclei during their preparation, Stoneburg (34) has indicated that special nuclear phospholipids exist. The significance of this constant equilibrium between the nuclear and cytoplasmic lipids is discussed later.

Regenerating Liver—In this group are three sets of rats (weights 209 to 232 gm.). Specific activities of urinary phosphates, as shown in Table II,

were followed in order to sacrifice animals when these values had reached a low level, since presumably they reflect values for tissue inorganic phos-

TABLE III

Specific Activities (Millimicrocuries per Mg.) of Phosphorus in Fractions of Tissue and of Nuclei

Experiment No	Group No	Days after injection	Whole tissue fractions					Nuclear fractions			
			Inorganic phosphate	Total protein	Lipid	Ribonucleic acid*	Thymonucleic acid	Total protein	Nucleic acid	Protein residue	Lipid
I Resting liver	1	3	103 5	62 0	158	56.9	11 1	.			
	2	3	121 0	57 5	120			18 2	14 8	17.3	117
	3	8	52.4	58 5	75 9	64 6	10 9				
	4	7	66 6	61 0	79.8			17 1	14 4	13.3	80.5
II Regenerating liver	5	3	81 9	167 0	150	188 0	147 0				
	6	13	27 4	96 4	37 1	86 2	158 0				
	7	16	25 7	82 1	24 8			162	175	178	29 6
III Hepatoma 31†	8	3	86 1	80 0	96 9	146 8	55 1				
	9	3	82 5	89 2	99 1			62.6	51 6	63 7	104 1

* Corrected for thymonucleic acid content.

† Specific activities have been corrected for the relatively smaller dose of P^{32} given, so that the values are comparable with those in Group 5

TABLE IV

Specific Activities of Nucleic Acid Phosphorus Expressed As Per Cent of Inorganic Phosphate Specific Activities

，	Group No	Days after injection	Ribonucleic acid	Thymonucleic acid
Resting liver	1	3	54 9	10 6
	3	8	123	20 8
Regenerating liver	5	3	230	180
	6	13	314	576
Hepatoma	8	3	171	64.0

phate. Comparison of Tables II and III will show that, in general, this is the case.

The specific activities of liver fractions are shown in Table III, Experiment II. Both nucleic acids are seen to have taken up a large amount of

P^{32} at the end of 3 days, so that their levels of specific activity greatly exceed that of inorganic phosphate. 13 to 16 days after injection, when the inorganic phosphate has reached a specific activity one-third of that on the 3rd day, the phosphorus activity of thymonucleic acid remains almost unchanged and is almost 6 times the inorganic level, while that of ribonucleic acid has dropped somewhat but remains 3 times the inorganic value. As before, nuclear proteins resemble the pure thymonucleic acid, and the nuclear phospholipids have fallen as if in free exchange with the more labile forms of phosphorus present in cells.

Hepatoma—Tumors were obtained from three rats (mean weight 353 gm.) sacrificed 72 hours after injection. As is seen in Table III, Experiment III, the specific activity of thymonucleic acid is 64 per cent of the inorganic value and the ribonucleic acid value is 171 per cent of the inorganic phosphate value. Nuclear proteins again resemble thymonucleic acid and nuclear phospholipids correspond to total phospholipids (Table III).

Since the tumor shows less P^{32} uptake by thymonucleic acid than does regenerating liver, it is of interest to compare their mitotic rates. The mean mitotic rate of regenerating liver over the period from 24 to 96 hours after partial hepatectomy is, from previous data (35), 1.2 per cent and represents a 115 per cent increase in total number of cells. A section of the hepatoma used in the experiment showed 0.6 per cent mitoses; allowing 1 hour for the duration of mitosis, this indicates a 54 per cent increase in 72 hours.

DISCUSSION

These results confirm the earlier impression (8, 36) that thymonucleic acid phosphorus is extremely stable in the non-growing organ. Since, in the first group of experiments, we were unable to maintain a constant activity of P^{32} in inorganic phosphate, and since the immediate precursor (12) of thymonucleic acid is not known, it is impossible to calculate the turnover rate. Inspection of the values does reveal, however, that if the precursor is inorganic phosphate the rate must be less than 11 per cent in 3 days (since inorganic activity is constantly declining), while if ribonucleic acid represents the precursor the rate cannot be much over 20 per cent in the same period. The failure of the phosphorus activity of thymonucleic acid to rise to meet that of the other measured fractions between the 3rd and 8th days further confirms its stability. A part of the P^{32} taken up by nuclear fractions may therefore represent a small, very labile moiety, as suggested by Marshak's observation (30) that considerable P^{32} appears in nuclear protein within 1 hour.

The stability of thymonucleic acid phosphorus is again emphasized in Experiment II (Table III). After thymonucleic acid has been built into

newly formed nuclei during growth, it then gives up its labeled phosphorus very slowly, so that it retains its high specific activity after the activities of labile forms of phosphorus have fallen to low levels. This observation seems to rule out the possibility that the apparent stability of the nuclear fractions in non-growing liver is due to breakdown of nucleic acid by the small amount of radiation present in these experiments.

The case of thymonucleic acid appears to be an exception to the postulate (37) that almost all reactions which the cell is able to carry out proceed at all times at rapid rates. The findings indicate that in the steady state existing in non-growing liver turnover of thymonucleic acid phosphorus is so slow that the reaction is far from completion in 1 or 2 weeks. In contrast to this, synthesis can occur in growing liver at a much more rapid rate. Thus, in the growing organ, either the rate of formation through the usual pathways is greatly accelerated, or a new pathway is utilized for synthesis.

It is clear from the data that ribonucleic acid turnover in resting liver, although much slower than that of phospholipids, greatly exceeds that of thymonucleic acid, and this must largely account for the high turnover of nucleoprotein reported by other observers (38, 39). It has been suggested (6) that ribonucleic acid is concerned with protein synthesis, which is a function of liver. The high P^{32} uptake by ribonucleic acid in regenerating liver probably represents both synthesis and turnover, and its considerable retention of P^{32} 2 weeks after partial hepatectomy suggests the possibility that part of this fraction may be relatively stable.

The turnover rate of phospholipids is so rapid that considerable fluctuations of tissue content can occur within this turnover through shifts in its equilibrium. This may well be true of other cell constituents which can be stored and discharged, for example proteins (37). Chargaff, Olson, and Partington (40) found that phospholipid turnover is depressed during regenerative growth, and suggested that this may be due to phosphorus mobilization for restorative tasks. The present findings show that synthesis of the nucleic acids may be an important competing reaction.

The remoteness of thymonucleic acid from the pool of labile cell phosphorus, seen in its normally slow turnover, would appear to stabilize it against daily fluctuations in amount, while it can be synthesized rapidly during cell proliferation. This apparently peculiar property of thymonucleic acid formation could insure stability of the cell as a unit and preservation of its genic material, and it is possible that the rate of its synthesis could be an important factor limiting the rate of tissue growth.

Hahn and Hevesy (36) have suggested that the low uptake of P^{32} by thymonucleic acid may be due to inability of phosphate to penetrate the nucleus. Against this is the apparently constant equilibrium between phospholipids of nucleus and cytoplasm which we observed (unless possibly

phosphorus enters the nucleus as phospholipid). Although Collip (41) concluded on histochemical grounds that phosphate is absent from the nucleus, the high concentration of phosphatases in hepatic nuclei (42) would seem to suggest that inorganic phosphate is present.

The low turnover of thymonucleic acid in the nucleus may be accounted for by assuming that it exists in the nucleus in highly polymerized form, as is indicated by the physical nature of the nucleoprotein extracted by Mirsky and Pollister (19). Thus, although phosphatases are present in high concentration, there may not at any moment be any considerable amount of substrate (oligo- or mononucleotides) on which they can act. The evidence presented by Mitchell (7) suggests that thymonucleic acid synthesis may occur directly from ribonucleotides. Such an interconversion between ribose and desoxyribose forms might occur without splitting of phosphate, through reduction of the sugar and methylation of uracil. It seems possible that the resulting change in physical properties of the nucleic acid formed might tend to stabilize the end-product.

SUMMARY

A method is outlined for the separation of thymonucleic and ribonucleic acids from small amounts of tissue. Analyses of preparations from resting and regenerating liver and hepatoma show that these ribonucleic acids resemble pancreas nucleic acid rather than yeast nucleic acid in their phosphorus and nitrogen content. Thymonucleic acid from Hepatoma 31 shows a phosphorus and nitrogen content equal to that of thymonucleic acid prepared from normal tissues.

The turnover of thymonucleic acid phosphorus in non-growing liver is extremely slow, in marked contrast to that of most cell constituents which have been investigated. After 3 days not over 11 per cent, and after 8 days not over 20 per cent of this fraction have exchanged radioactive phosphorus with tissue inorganic phosphate. During regenerative growth large amounts of radioactive phosphorus are acquired by thymonucleic acid, apparently due to newly synthesized material. Radioactive phosphorus thus built into newly formed nuclei during the growth period is then held in high concentration over at least a 2 week period, confirming the slowness of its turnover.

Hepatoma 31, which has a growth rate intermediate between that of resting and regenerating liver, shows an intermediate rate of radioactive phosphorus uptake by thymonucleic acid.

Ribonucleic acid turnover in non-growing liver is considerably slower than that of phospholipids but is considerably more active than that of thymonucleic acid.

Nuclear and cytoplasmic phospholipids at all times appear to be in

equilibrium, suggesting that slow thymonucleic acid turnover is not due to exclusion of phosphorus from the nucleus during the resting stage.

The implications of this metabolic peculiarity of thymonucleic acid metabolism (low turnover, but the potentiality of rapid synthesis) with regard to cell growth and nuclear stability, and its possible mechanisms, are discussed.

BIBLIOGRAPHY

1 Schultz, J., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **9,** 55 (1941)

2 Astbury, W T , and Bell, F , *Nature,* **141,** 747 (1938).

3 Darlington, C D , *Nature,* **149,** 66 (1942).

4 Stedman, E , and Stedman, E., *Nature,* **152,** 267 (1943).

5. Claude, A., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **9,** 263 (1941)

6. Caspersson, T., and Schultz, J., *Proc Nat Acad Sc ,* **26,** 507 (1940).

7. Mitchell, J S , *Brit J Exp. Path ,* **23,** 285 (1943)

8 Brues, A M , Tracy, M M , and Cohn, W E , *Science,* **95,** 558 (1942).

9 Mirsky, A E., in Nord, F F , and Werkman, C H , Advances in enzymology and related subjects, New York, **3,** 1 (1943).

10. Straub, J , *Naturwissenschaften,* **31,** 97 (1943)

11. Koller, P C , *Nature,* **151,** 244 (1943)

12 Zilversmit, D B , Entenman, C , and Fishler, M C., *J Gen. Physiol ,* **26,** 325 (1943).

13. Chaikoff, I. L , *Physiol Rev ,* **22,** 291 (1942)

14. Higgins, G M., and Anderson, R M , *Arch Path ,* **12,** 186 (1931).

15. Fiske, C H., and Subbarow, Y , *J Biol Chem ,* **66,** 375 (1925)

16. Pugsley, L I., *J Physiol ,* **76,** 314 (1932)

17. Brues, A M , Drury, D. R , and Brues, M C , *Arch. Path ,* **22,** 658 (1936).

18. Huiskamp, N , *Z physiol Chem ,* **32,** 145 (1901)

19. Mirsky, A. E , and Pollister, A W., *Proc Nat Acad Sc.,* **28,** 344 (1942).

20. Hammarsten, E , *Biochem Z ,* **144,** 383 (1924)

21. Bang, I , *Beitr. chem Physiol. u Path ,* **1,** 189 (1902); **4,** 115 (1904)

22. Jorpes, E., *Acta med Scand.,* **68,** 503 (1928)

23. Levene, P A., and Bass, L W , Nucleic acids, American Chemical Society monograph series, New York, 298 (1931).

24. Feulgen, R , Behrens, M , and Mahdihassan, S , *Z physiol. Chem ,* **246,** 203 (1937).

25. Jones, W. E , and Perkins, M. E., *J Biol Chem ,* **62,** 291 (1924-25).

26. Calvery, H. O , *J Biol. Chem ,* **77,** 489 (1928)

27. Cohen, S. S , and Stanley, W M., *J. Biol. Chem ,* **144,** 589 (1942).

28 Levene, P A., and Jorpes, E , *J Biol Chem ,* **86,** 389 (1930).

29. Seibert, F. B , *J. Biol Chem.,* **133,** 593 (1940).

30. Marshak, A , *J. Gen. Physiol.,* **25,** 278 (1941)

31. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
32. Davidson, J. N., and Waymouth, C., *Nature*, **152**, 47 (1943).
33. Stern, K., and Willheim, R., *Biochem. Z.*, **272**, 180 (1934).
34. Stoneburg, C. A., *J. Biol. Chem.*, **129**, 189 (1939).
35. Brues, A. M., and Marble, B. B., *J. Exp. Med.*, **65**, 15 (1937).
36. Hahn, L., and Hevesy, G., *Nature*, **145**, 549 (1940).
37. Schoenheimer, R., *Growth, 2nd growth symposium*, **4**, 27 (1940).
38 Tuttle, L E., Erf, L. A., and Lawrence, J. H., *J. Clin. Invest.*, **20**, 57 (1941).
39. Kohman, T. P., and Rusch, H P., *Proc. Soc Exp. Biol. and Med.*, **46**, 403 (1941).
40. Chargaff, E., Olson, K. B., and Partington, P. F , *J Biol. Chem.*, **134**, 505 (1940).
41. Collip, J. B , *J. Biol. Chem.*, **42**, 227 (1920).
42. Dounce, A. L., *J. Biol. Chem.*, **147**, 685 (1943).

FACTORS INFLUENCING THE RIBOFLAVIN CONTENT
OF THE CORNEA*

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A number of reports indicate that mild degrees of corneal vascularity are highly prevalent among sailors, aviators, and others exposed as a result of their activities to excessive sunlight, snow reflection, salt spray, blowing sand, and other such environmental factors (1–3).[1] Since ariboflavinosis is among the causes which can lead to corneal vascularity, it has been suggested that riboflavin deficiency may be responsible for these lesions. Furthermore, since the lesions occur in many who would be considered well nourished by our present standards, it has been suggested that excessive exposure to strong light serves to accentuate the riboflavin requirements (1–3).[1] It is well known that riboflavin is sensitive to destruction by light. This sensitivity suggests the possibility that tissues, such as the cornea, which are exposed to light, might at times undergo local riboflavin deficiencies in spite of the presence of adequate dietary riboflavin for the rest of the body. These considerations would seem to justify further investigation. Furthermore, since signs of riboflavin deficiency become apparent in the cornea at an early stage, it would be of value to have information on the concentration of riboflavin in this tissue relative to both dietary intake and appearance of vascularity.

It would be difficult to prove in man whether or not such changes are due specifically to a local decrease in riboflavin. Therefore the studies reported below have been made on the rat to determine (a) the influence of different levels of riboflavin intake on the riboflavin concentration of the cornea, and (b) whether light will affect the riboflavin concentration in the cornea. On the same animals observations have been made relative to the influence of light and riboflavin concentration on the development of the typical corneal lesion of riboflavin deficiency. These will be published in a separate paper.

Primarily, the cornea consists of two thin layers of living cells and a thick, much less cellular layer of stroma. Thus the active cytoplasm is not evenly

[1] Kruse, H. A., personal communication.

distributed throughout the cornea but is concentrated in the two cellular layers. Since the outer layer of cells, the corneal epithelium, is the thicker of the two cellular layers, it may be expected to contain the bulk of the cells of the cornea and hence the bulk of the substances characteristic of living cells. Riboflavin is one of these substances. That the corneal epithelium actually contains most of the corneal riboflavin has been demonstrated by direct measurements made during the present investigation. This confirms a recent observation made on ox corneas by Philpot and Pirie (4).

The riboflavin content of the whole cornea would therefore be affected, not only by its concentration in the living epithelial cells, but by the proportion of living cells to non-cellular or extracellular tissue. It is conceivable that as a result of riboflavin deficiency itself, or as a result of trauma from light, a change might occur in the amount of epithelial cells present. Such a change would by itself materially affect the riboflavin content of the cornea as a whole.

An attempt was therefore made to estimate the relative proportion of cells in the corneas examined. The concentration of total phosphorus is 30 or 40 times greater in most cells than it is in extracellular fluid or inert structures such as collagen fibers. The phosphorus content of a tissue may therefore be used as an approximate index of cellularity. To this end, the phosphorus content of the cornea has been measured and used in addition to dry weight as the quantitative basis for the analyses reported below. By the use of a recently developed micromethod for the determination of riboflavin in small quantities of tissue, the riboflavin concentration of the corneas of rats maintained for varying periods on varying levels of riboflavin has been determined. Likewise, the analyses of single corneas from rats maintained for varying periods in light of different intensities have been compared with controls left in the dark.

<div style="text-align:center">EXPERIMENTAL</div>

Albino rats of the Wistar strain, 45 to 55 gm. in weight at the start of the experiment, were used in all experiments except the ultraviolet irradiation studies; in this case the animals weighed 125 to 150 gm. Except where otherwise stated, the animals received a diet composed of vitamin-free casein 18 per cent, dextrose 20 per cent, corn-starch 51 per cent, peanut oil 8 per cent, and Osborne and Mendel's salt mixture (5) 3 per cent. In addition, vitamin supplements were given by stomach tube three times a week to furnish an average daily intake of 21 γ of thiamine, 21 γ of pyridoxine, 42 γ of calcium pantothenate, and an aqueous riboflavin-free extract of rice polishings equivalent to 0.43 gm. of dry rice polishings. The control animals received riboflavin similarly administered. However, in studies of the influence of different levels of riboflavin intake on the corneal riboflavin,

the riboflavin was given daily. Corneas were removed for analysis 16 to 18 hours after the last dose of riboflavin.

The influence of light on the corneal riboflavin was studied by placing rats in cages continuously illuminated with four 75 watt incandescent lamps placed about 7 inches from the center of the cage. Sufficient light reached the rats' eyes to cause a mild conjunctivitis continuously throughout the experiments. A uniform temperature was maintained by rapidly circulating air. Control animals were maintained in the same room but in darkness.

The more severe treatment with light was supplied by irradiation of the cornea with a mercury arc lamp (General Electric No. HB-4), a rich source of ultraviolet light. Anesthetized rats were given different doses of irradiation. The highest doses were severe enough to produce irreparable damage to the cornea. The irradiated eye was held open with adhesive tape bound around the head. The other eye was bound shut meanwhile and served as a control. The corneas were analyzed separately for riboflavin. The corneas were removed either immediately following the ultraviolet exposure or after the rats had been maintained for different intervals in darkness or surrounded by four 75 watt incandescent lamps.

The procedure used for removing the cornea for chemical analysis was as follows: The protruded eyeball was held in a hemostat clamped behind the eye on the periorbital tissues, and the eyeball cut free, briefly rinsed in isotonic saline, lightly dried with hardened filter paper, and immersed in a mixture of solid CO_2 and petroleum ether. The cornea was then easily removed from the frozen eyeball by cutting close to the limbus with a scalpel and shelling off the frozen corneal disc. After thawing, it was blotted gently with hardened filter paper and placed in the bottom of a 0.75 ml. serological tube which had been previously weighed on a micro balance. A number of these tubes were placed together in a larger test-tube and frozen-dried by (a) dipping the larger tube in a petroleum ether-solid CO_2 mixture, (b) establishing a vacuum with an oil pump, and (c) removing the tube from the freezing mixture and maintaining the vacuum for 45 minutes. The dry weight of the cornea (0.75 to 1.5 mg.) was then obtained with the micro balance.

The riboflavin content of the cornea was determined with a microbiological method which has been previously described (6). For this purpose the corneas were autoclaved in 0.1 N HCl and then partially neutralized with alkali and centrifuged. After three aliquots of the supernatant fluid were removed for riboflavin determination, there remained a little supernatant fluid. Aliquots of this were used for the phosphorus determination.

In more than half of the cases in which both of the eyes had received identical treatment, the corneas were analyzed separately. Under these circumstances the difference between the riboflavin concentration in the

two corneas of the same rat was found to have a coefficient of variation of 4 per cent. In some instances the two corneas were pooled and analyzed as one sample.

A colorimetric molybdic acid method was used for measuring the phosphorus content. The aliquots (0.025 ml., representing 0.04 to 0.08 mg. of cornea) were transferred with Levy-Lang constriction pipettes (7) to the bottom of special Pyrex tubes 10 cm. in length with an inner diameter of 4 mm. To each tube was added 0.035 ml. of 10 N H_2SO_4 and the water was allowed to evaporate overnight in an oven at 95°. The charred samples were then wet ashed by adding to each a 0.01 ml. drop of fuming nitric acid and heating for 15 minutes in an oven at 130°. The addition of fuming nitric acid was repeated once, and after being heated for an hour longer at 130° the tubes were ready for color development. To each tube was added 0.7 ml. of a reagent which was freshly made by dissolving 0.6 gm. of the dry reducing mixture described by Fiske and Subbarow (8) in 90 ml. of water and adding 10 ml. of 2.5 per cent molybdic acid in 5 N sulfuric acid. The tubes were thoroughly mixed with a slender, spiral, glass rod, heated for 90 minutes at 60°, and read in a Beckman spectrophotometer at a wavelength of 820 mμ. In order to read a volume of 0 7 ml. in the standard cells of the instrument, a horizontal 1 mm. slit was inserted in the path of the light beam at a point just before the light enters the cell The cells were raised on blocks as far as possible without interfering with the narrow beam of light. This permitted as little as 0.4 ml. to be read in the instrument without sacrifice in accuracy. Approximately 8 times as much color is obtained by the combination of heating and reading at 820 mμ as with the original Fiske and Subbarow procedure when read at 650 mμ. As little as 0.08 γ of P could be determined, as described above, with a precision of 2 or 3 per cent This is the P content of 0 05 mg. of cornea or 0.02 mg. of liver.

The color development was carried out at 60° instead of 95–100° to increase the consistency of the results. At 100° occasional tubes failed to develop full color. This appeared to be due to an excessive loss of SO_2 combined with reoxidation of the reduced molybdate by air. To minimize both of these effects further, the heating was carried out in the long slender tubes described above, which increased the fluid depth.

Results

The corneal epithelium contains 6 or 8 times as much riboflavin per gm. as the rest of the cornea (Table I). The epithelium was scraped off the cornea with a scalpel and the epithelium from both corneas was analyzed as one sample. The phosphorus concentration almost exactly parallels the riboflavin concentration. Therefore the amount of riboflavin per mole of phosphorus is nearly the same in the epithelium as in the rest of the cornea.

This supports the suggestion that phosphorus is a more reliable basis than dry weight for measuring changes in the riboflavin concentration in tissues. Some of the epithelium was certainly lost during the scraping process, but the amount of epithelium actually isolated contained 64 per cent of the total in the cornea, a lower limit.

Riboflavin values for normal young rats on a dog chow diet are also shown in Table I, together with data for rats 7 weeks on the riboflavin-deficient diet. It will be seen that the decrease in corneal riboflavin on the deficient diet is not accompanied by a fall in phosphorus. This makes it probable that there is a real decrease in the riboflavin concentration in the cells.

TABLE I

Riboflavin and Phosphorus Concentration in Corneal Epithelium, Stroma, and in Entire Cornea

Diet	Time on diet	No of rats	Part of cornea	Dry weight	Riboflavin	P	Riboflavin
	wks			*mg*	γ *per gm*	*mM per kg.*	*mg per mole P*
Dog chow		1	Epithelium	0.345	9.04	228	40
			Stroma	1.240	1.44	40	36
" "		1	Epithelium	0.280	9.35	196	48
			Stroma	1.212	1.29	·32	41
" "	1–2	7	Entire		5.47	115*	47*
Riboflavin-free	2	8	"		2.94	120	24

* Based on four rats.

The cornea promptly reflects changes in dietary riboflavin (Fig. 1). The data from 102 rats are represented in Fig. 1, each point representing the average of three to eight animals. A distinct fall in riboflavin concentration is observed within 3 days after rats are placed on a riboflavin-free diet. The fall is more rapid at first and then levels off after 3 weeks at approximately 40 per cent of its initial value. It is about this time that deficiency signs appear in the cornea. With less than optimum intakes of riboflavin (*i.e.*, 21 γ daily) there is a slower fall, which eventually nearly reaches the deficient level. Apparently during the first 3 weeks at least, 40 γ of riboflavin per day provide as much of this vitamin as the cornea can use, whereas 21 γ per day are inadequate to maintain the corneal riboflavin at the highest level possible. In spite of the highest amounts of riboflavin administered, 160 γ per day, there was a fall of approximately 10 per cent in the corneal riboflavin by the end of 21 days. The slow decline in the 21 γ curve after 3 weeks we interpret as being due to the increased requirements of these growing animals.

The administration of 250 γ of riboflavin per day to rats which had been

on a riboflavin-free diet for 16 weeks resulted in a prompt rise in corneal riboflavin (Fig. 1). One eye of each rat was removed for a control analysis just before the first riboflavin was administered. Within 24 hours there was an increase in riboflavin of nearly 100 per cent in the cornea of the remaining eye, and by 48 hours the recovery seemed to be complete. It is perhaps significant that in spite of the large amounts of riboflavin employed, the cornea never fully regained the riboflavin concentration of rats continuously maintained on high riboflavin diets. The results of this recovery experiment are in accord with observations made on other tissues by Ochoa and Rossiter (9).
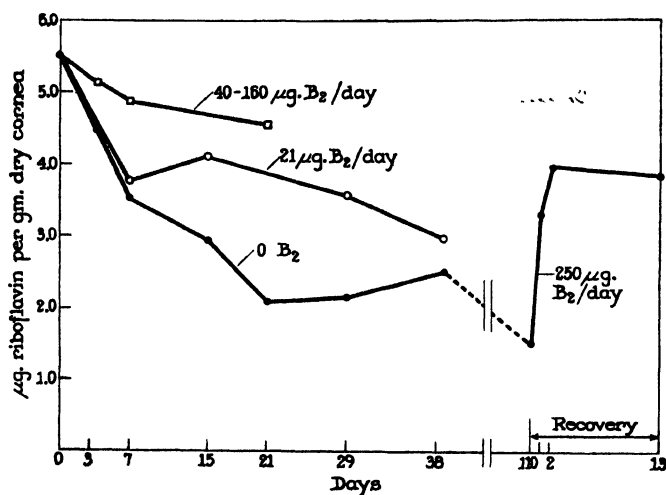
Fig. 1. Effect of time and riboflavin intake on corneal riboflavin

The continuous maintenance of rats in bright light proved to be without demonstrable influence on the corneal riboflavin (Table II). The corneas were analyzed after 8, 16, 29, and 38 days in the light. The results have been averaged together. Neither deficient rats nor control rats on a moderate riboflavin intake (21 γ daily) showed any significant influence of light on the riboflavin content of the cornea. ·This was true whether dry weight or phosphorus was used as a basis of calculation.

Like visible light, ultraviolet light failed to lower the riboflavin concentration in the cornea (Table III). Only one eye of each rat was irradiated; the opposite eye served as a control. The percentage change between irradiated and non-irradiated corneas is recorded (Table III). With repeated

irradiation there seems to be actually a slight increase in riboflavin, but this increase is accompanied by an increased phosphorus content and probably is the result of leucocytic infiltration, since many of these corneas were edematous, turbid, and invaded by capillaries.

TABLE II

Corneal Riboflavin of Rats Maintained in Bright Light for 8 to 38 Days

	Riboflavin	No. of rats	Riboflavin*	P*	$\frac{\text{Riboflavin}}{\text{P}}$
	γ *per day*		γ *per gm.*	*mм per kg*	*mg. per mole*
Initial .		7	5.47	115	47
Light	0	10	2.70	116	23
Dark	0	10	2.86	123	23
Light	21	10	3.75	121	31
Dark	21	10	3.65.	114	32

* Based on dry weight.

TABLE III

Effect of Ultraviolet Light on Corneal Riboflavin

Length of irradiation	No of irradiations	Time after last irradiation	No of rats	Change in irradiated eye compared to control eye		
				Riboflavin	P	$\frac{\text{Riboflavin}}{\text{P}}$
min		*hrs.*		*per cent*	*per cent*	*per cent*
15	1	0	3	−7	−3	−4
15	1	4	5	−5	−2	−3
15	1	20	3	+1	−12	+12
5*	6†	20	2	+16	+17	−2
10*	6†	20	2	+18	+17	+1
15‡	6†	20	2	+8	+18	−8
30‡	6†	20	2	+8	+2	+6
Average .			19	+2	+2	0
" riboflavin-deficient only..			6	+4	+7	−4

* Rats maintained in bright light (incandescent lamps) between periods of ultra-violet exposure.
† At 24 hour intervals.
‡ Rats maintained in darkness when not being irradiated with ultraviolet light.

Some of the rats irradiated with ultraviolet light were on a diet deficient in riboflavin. There was no demonstrable effect of ultraviolet light on the corneal riboflavin. These findings are in agreement with those of Philpot and Pirie (4) who irradiated ox corneas with ultraviolet light *in vitro*.

The concentration of riboflavin in the cornea may be varied over a wide range. Indeed, it is only when the concentration falls to less than half of the maximum value that visible signs of deficiency appear in the cornea. This raises the difficult question of whether there is any advantage to the cornea of more than the minimum amount of riboflavin to prevent symptoms. With an amount of riboflavin (21 γ per day) which permitted the level of riboflavin to fall to 50 or 60 per cent of the maximum, there were no visible signs in the cornea of deficiency even in the case of rats with the additional stress of continuous illumination with visible light. It would, of course, be of advantage to have a reserve of riboflavin in any tissue, since this would permit the animal to undergo periods of deficiency without the development of symptoms.

It is probable that most of the corneal riboflavin is in combined form. The insensitivity to ultraviolet light supports this belief. There seems to be an upper limit to the amount of riboflavin which the cornea can retain. It is reasonable that this upper limit is determined by the amount of riboflavin "acceptors" present or, since most of the riboflavin is probably present as the flavin adenine dinucleotide (9), by the dinucleotide "acceptors." These acceptors might be either flavin enzymes or other, less specific, cellular constituents. Since the deficient cornea very promptly accepts riboflavin when flavin again becomes available, either the riboflavin acceptors are still present or are very rapidly synthesized. However, the cornea does not accept quite as much riboflavin as was present initially. Perhaps this means that some of the riboflavin acceptors have been permanently lost. In a study of rat liver in riboflavin deficiency, Rossiter (10) found little change in the concentration of the protein portion of the flavin enzyme d-amino acid oxidase in spite of a greatly decreased activity resulting from a deficiency in the prosthetic group. There are, of course, many other flavin enzymes present in tissues which might be decreased during a prolonged deficiency.

The failure of the severe treatment of the cornea with ultraviolet light to affect the concentration of riboflavin suggests that the amount of illumination experienced by persons exposed for considerable periods to bright sunlight will not affect the riboflavin already present in the cornea. There remains the possibility that riboflavin as it diffuses into the cornea, presumably in a free form, may be destroyed by light on the way in. The failure to observe an influence in rats of continuous rather intense illumination on the corneal riboflavin suggests that such destruction, if it occurs, is incomplete and without detriment to the cornea. While strong light will lead to corneal vascularity, as shown by this study, it seems highly improbable that this tissue reaction has any direct relation to riboflavin nutrition.

SUMMARY

1. A procedure for the determination of phosphorus in 0.05 mg. of cornea is described.

2. The riboflavin and phosphorus of the cornea are found to be largely concentrated in the epithelium.

3. The concentration of riboflavin in the cornea is promptly influenced by the level of riboflavin intake. 40 γ per day produce a maximum effect at least up to 3 weeks. With lesser amounts of riboflavin the concentration of corneal riboflavin falls, whether calculated on the basis of dry weight or on the basis of phosphorus present.

4. There is no demonstrable influence of light, either visible or ultraviolet, on the concentration of corneal riboflavin.

BIBLIOGRAPHY

1. Tisdall, F. F , McCreary, J. F., and Pearce, H., *Canad. Med. Assn. J.*, **49,** 5 (1943).
2. Tisdall, F. F., Proceedings, 20th annual conference, Milbank Memorial Fund, New York, May 7, 1942, 35.
3. Youmans, J B , Patton, E. W., Robinson, W. D., and Kern, R., *Tr. Assn. Am. Phys.*, **57,** 49 (1942).
4. Philpot, F. J , and Pirie, A., *Biochem. J.*, **37,** 250 (1943).
5. Osborne, T B , and Mendel, L B , *J Biol Chem.*, **37,** 572 (1919).
6. Lowry, O. H., and Bessey, O. A., *J Biol Chem.*, **155,** 71 (1944).
7. Levy, M , *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **21,** 101 (1936).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
9 Ochoa, S , and Rossiter, R J , *Biochem. J* , **33,** 2008 (1939).
10. Rossiter, R. J., *J. Biol. Chem* , **135,** 431 (1940).

ON THE MECHANISM OF THE CONVERSION IN VIVO
OF METHIONINE TO CYSTINE

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Since the demonstration of the metabolic conversion of methionine to cystine, the literature has been replete with theories and speculations concerning the mechanism of this conversion. Some of these suggested mechanisms have been based on *in vitro* analogies, some on indirect metabolic evidence, and others on no apparent experimental basis. Obviously, all mechanisms involve the utilization of the sulfur of methionine for the synthesis of cystine, a fact which has been indubitably established with the use of radioactive sulfur (1). Beyond this common ground, the theories may be considered in two categories; they postulate either (*a*) the utilization of the carbon chain of methionine in the conversion to cystine or (*b*) only the utilization of the sulfur. The three postulated mechanisms which fall into the latter category include that of Nicolet (2) in which cystine formation results from the interaction of H_2S with aminoacrylic acid, that of Brand, Block, Kassell, and Cahill (3) in which homocysteine reacts with aminoacrylic acid to yield a thio ether which cleaves to form cystine, and that of Toennies (4) in which methionine reacts with a hydroxy-amino acid to form a sulfonium compound which splits off methyl alcohol and eventually cleaves to yield cystine. On the other hand, there have been two theories suggested in which the carbon chain of methionine is shortened to yield the 3-carbon chain of cysteine, in one case by demethylation to homocysteine followed by an oxidative degradation at the amino-carboxylic end of the chain as postulated by du Vigneaud and collaborators (5), and in the other by a shift of the sulfur followed by ω oxidation and decarboxylation, as suggested by Tarver and Schmidt (1).

It is the purpose of the present paper to establish definitely whether the carbon chain of methionine is utilized in the conversion to cystine in the animal body. This was accomplished by feeding white rats on a cystine-free diet with methionine labeled with both isotopic sulfur and isotopic carbon, the latter in the β and γ positions, and subsequently isolating the cystine from the hair of these animals for the determination of its isotopic content.

In an earlier investigation[1] we made an attempt to solve this problem by

[1] du Vigneaud, V., Dyer, H. M., Cohn, M., and Brown, G. B., unpublished data.

feeding methionine whose carbon chain was labeled by means of deuterium. It was realized that this approach could be decisive only if the cystine isolated after the feeding of the compound contained deuterium because of the possibility of the labilization of the deuterium during a conversion of methionine to cystine. However, it was deemed worth while at least to carry out exploratory experiments in this direction. Dideuteriomethionine with the deuterium attached in the β and γ positions was synthesized (6). The cystine actually isolated after the feeding of the dideuteriomethionine contained no deuterium. This result did not permit a unique interpretation and required further investigations of the possible labilization of the deuterium. Further work along this line was initiated but was abandoned when the carbon isotope became available for labeling the carbon chain directly.

Since that time the application of different methods of approach has yielded evidence concerning certain aspects of the mechanism of the conversion of methionine to cystine. From studies of the behavior of the thio ether, cystathionine, both in the intact animal (7) and tissue slices (8, 9), the mechanism which seems most strongly supported by experimental evidence at the moment is a modification of the hypothesis of Brand, Block, Kassell, and Cahill. The first step in the proposed mechanism is the demethylation of methionine to homocysteine. It now appears that serine interacts with the homocysteine to form cystathionine, the latter cleaving to form cysteine. Both tissue slice experiments from this laboratory (9) and a study with N^{15} by Stetten (10) indicate that serine is the precursor of the carbon chain of cysteine and cystine.

The present study in which both the sulfur and the carbon are labeled demonstrates conclusively that the carbon chain of methionine is not converted to cystine. Although approximately 80 per cent of the sulfur in the isolated cystine had been derived from fed methionine, no significant amount of the carbon in the cystine originated in the methionine fed. These results preclude acceptance of any mechanism involving the conversion of the carbon chain of methionine to the carbon chain of cystine in the animal body.

<center>EXPERIMENTAL</center>

The synthesis of the labeled methionine has been described in a previous paper (11). The sample fed contained 4.23 atom per cent excess S^{34} and 1.67 atom per cent excess C^{13}. The isotopic carbon was in the β and γ positions.

Feeding Experiment—Four young rats, all litter mates, of about 60 gm. weight, were used. The rats were fed a basal diet in which the amino nitrogen was supplied by pure amino acids in a mixture patterned after that of

Rose and Rice (12). The basal diet had the following percentage composition: amino acid mixture 21.3² (13), sâlt mixture 4.0 (14), hydrogenated vegetable oil (Crisco) 30.0, sucrose 44.7. The oil-soluble vitamins were included in the Crisco as halibut liver oil, irradiated ergosterol in cottonseed oil, dl-α-tocopherol, and menadione, and provided 4000 U. S P. units of vitamin A, 400 U. S. P. units of vitamin D, 1.0 mg. of vitamin E, and 100 γ of vitamin K per 100 gm. of diet. The water-soluble vitamins were administered by pipette as 1 ml. of an aqueous solution providing the rats daily with 20 γ of thiamine hydrochloride, 20 γ of riboflavin, 20 γ of nicotinic acid, 20 γ of pyridoxine hydrochloride, 100 γ of dl-calcium pantothenate, 5 mg. of inositol, and 25 mg. of choline-free ryzamin-B.

The rats were permitted to consume the basal diet ad libitum for a period of 3 days during which they lost weight. On the 4th day, the basal diet was supplemented with 0.6 per cent methionine, ordinary methionine for Rats 827 and 828, and isotopic methionine for Rats 825 and 826. At the end of the 4th day, the animals³ were anesthetized with ether and dehaired with the use of a depilatory. The level of methionine in the diet was continued at 0.6 per cent for 12 days. At the end of this period, the methionine content of the diets of the control and experimental animals was lowered to 0.5 per cent. The animals were consuming such large quantities of food per day that it was necessary to lower the percentage of methionine in the diet further to 0.43 per cent on the 18th day, and again to 0.38 per cent on the 24th day. Furthermore, on the 28th day, the total food consumption per day was limited to 8 gm. per rat. At the end of the 38 days, all the animals were returned to the basal diet for 2 days. All the rats were then dehaired by clipping the hair as closely as possible to the skin. The data for the feeding experiments are summarized in Table I.

Isolation of Cystine—500 mg. of the thoroughly washed and degreased hair were hydrolyzed under a reflux for 20 hours with 15 ml. of a 1:1 hydrochloric acid-formic acid mixture. The mixture was refluxed at 100° for about 1 hour and then the temperature was raised to 125°. At the end of 20 hours, the hydrolysate was evaporated to dryness and the residue was dissolved in 10 ml. of 1 N HCl. The procedure that followed, to the stage of separation of the cuprous mercaptide, was essentially the method employed by Graff, Maculla, and Graff (15) for the estimation of the cystine content of tissue proteins. The washed cuprous mercaptide was decomposed by

² The apparent percentage of amino acid mixture in the diet used in this paper differs from that previously described because the present diet contains 3.8 per cent dl-lysine hydrochloride rather than 4.5 per cent dl-lysine dihydrochloride and 2.3 per cent sodium bicarbonate rather than 3.9 per cent.

³ One of the control animals was killed in the dehairing process and a litter mate, Rat 827, was substituted for it. As a result of the accident, this animal was fed the 0.6 per cent methionine diet for 5 days less than the other three animals.

passing H_2S through the hot acidified suspension of cuprous mercaptide for about 2 hours. The suspension was then centrifuged, the supernatant liquid was decanted, and the sludge of Cu_2S was washed with 0.1 N HCl. The supernatant liquid and washings were warmed to remove any H_2S and evaporated to a volume of about 5 ml. The cysteine was oxidized to cystine with a dilute alcoholic solution of iodine, excess iodine being avoided. The solution was evaporated to dryness and the residue was dissolved in 2 ml. of water and brought to pH 4.5 with 0.5 N NH_4OH solution. The solution was allowed to stand overnight in the ice box and the cystine obtained was recrystallized by dissolving in 0.1 N HCl and adjusting the pH to 4.5 with 0.5 N NH_4OH solution.

A preliminary isolation from ordinary rat hair indicated that the cystine from the first crystallization was 98 ± 5 per cent pure by polarographic analysis. The isolated cystine had a specific rotation of $[\alpha]_D^{22} = -143°$ in 1 N HCl. The yields of cystine from the hair of the experimental animals

TABLE I

Feeding of Methionine

Rat No.	Duration of experiment	Change in body weight	Total methionine ingested
	days	*gm*	*gm.*
825	35	53–114	1.17
826	35	54–120	1.18
827*	30	69–123	1.07
828	35	58–120	1.07

* See foot-note 3.

were less than those obtained in the preliminary isolations with ordinary rat hair. A polarographic analysis of the hydrolysate of ordinary rat hair showed that cystine was present in the hair at an approximate concentration of 12 per cent. A similar analysis for one of the control animals (Rat 828) indicated a lower cystine content, namely 10 per cent, which may be ascribed to the low sulfur content of the diet in these experiments, as previously pointed out by other investigators (16, 17).

Elementary analyses of the recrystallized cystine from the hair of the control animals and from ordinary rat hair showed that the product was analytically pure. The cystine samples from Rats 825 and 826 were not analyzed in order to conserve the valuable isotopic products.

Analyses—$C_6H_{12}O_4N_2S_2$

	Calculated.	C 29.99,	H 5.03
Ordinary hair.	Found.	" 30.12,	" 5.06
Rat 827.	"	" 30.16,	" 4.99
" 828.	"	" 29.96,	" 5.18

Determination of Isotopic Content—For the purpose of determining the isotopic ratios of the fed and isolated compounds, the carbon and sulfur had to be converted to a form which could be readily analyzed with the mass spectrometer. For the determination of the carbon, 1 to 2 mg. of the methionine or cystine was burned to CO_2 in a stream of oxygen in a micro combustion tube packed only with CuO wire. The products of combustion were passed through a gas scrubber containing 5 ml. of a clear saturated $Ba(OH)_2$ solution. The precipitated $BaCO_3$ was centrifuged, washed, and transferred to an asbestos mat in a small perforated glass cup. The $BaCO_3$ was reconverted to CO_2 by treatment with dilute HCl in a conversion apparatus similar to that used by Rittenberg, Keston, Rosebury, and Schoenheimer for the liberation of nitrogen (18).

In the case of sulfur, it was found more satisfactory to reduce the compound to H_2S rather than oxidize to SO_2. For this purpose 4 to 5 mg. of

TABLE II

Isotopic Analysis of Fed Methionine and of Isolated Cystine

All samples were analyzed on the same day and the S^{34} and C^{13} contents of the control isolated cystine were assumed to be the normal abundance in calculating the excess for methionine and cystine.

Compound	Sulfur analysis		Carbon analysis	
	S^{34}	S^{34} in excess	C^{13}	C^{13} in excess
	atom per cent	*atom per cent*	*atom per cent*	*atom per cent*
Control isolated cystine (Rat 827)	4.50	0.00	1.18	0.00
Fed methionine	8.73	4.23	2.85	1.67
Isolated cystine (Rat 825)	7.96	3.46	1.20	0.02
" " (" 826)	7.90	3.40	1.19	0.01

methionine or cystine were heated in a stream of hydrogen in a micro combustion tube that was packed only with platinum gauze maintained at approximately 700°. The gases were passed through a scrubber containing about 5 ml. of a solution of 15 per cent zinc acetate in 10 per cent sodium acetate. The zinc sulfide obtained was centrifuged, washed, and collected in the same manner as the $BaCO_3$. Attempts to obtain maximum yields of H_2S from the ZnS finally led to the use of 100 per cent phosphoric acid in the conversion process. The values of the isotopic ratios for the carbon and sulfur of the methionine fed and the cystines isolated are listed in Table II.

DISCUSSION

The experimental results obtained, in so far as the sulfur is concerned, agree qualitatively with those previously obtained by Tarver and Schmidt

(1) in their experiments with radioactive sulfur. The discrepancy in the quantitative aspects of the results, *i.e.* the higher percentage of isotopic sulfur found in the isolated cystine in our experiments, may be due to the differences in experimental conditions, all of which would tend to make the results diverge in the direction found. The present experiments were designed to attain a maximum amount of conversion of methionine to cystine. Whereas the diet in our experiments contained only isotopic methionine, that of the previous investigators contained approximately 0.2 per cent ordinary methionine in addition to 0.15 per cent labeled methionine. Another difference in the diet was the complete absence of cystine in the present experiments, while the diet in the experiments of Tarver and Schmidt contained approximately 0.02 per cent cystine, of the order of 2 mg. per day. It should also be pointed out that in the same period of time our animals gained 60 gm. compared to 40 gm. in the other experiments, our diet containing the equivalent of approximately 3 times their protein concentration.

Since the isotopic sulfur content of the isolated cystine was 80 per cent of that in the fed methionine, there can be no question that, had the carbon of methionine been converted to cystine, it would have been detected in significant quantity. If the 2 labeled carbon atoms of methionine had been transferred to the cystine intact with the sulfur, 80 per cent of 2 carbon atoms in the cystine isolated would have been derived from the methionine. Since the initial value of the carbon in the methionine was 1.67 atom per cent excess C^{13} and the number of carbon atoms is decreased from 5 to 3, the C^{13} in the isolated cystine from the hair would have been $0.80 \times 1.67 \times 5/3$ or 2.2 atom per cent excess. If the degradation of the methionine chain took place in such a way that only 1 of the labeled carbon atoms was retained, one-half of this quantity or 1.1 atom per cent excess C^{13} would be expected in the isolated cystine. Thus, in either case, a utilization of the carbon chain of methionine for the synthesis of cystine in the animal body could have been readily observed under the present experimental conditions and it may be concluded that no appreciable conversion of the carbon chain of methionine to cystine occurs.

SUMMARY

Methionine labeled with S^{34} and with C^{13} in the β and γ positions was fed to rats and the cystine was subsequently isolated from the hair. It was found from isotopic analysis of the cystine that approximately 80 per cent of its sulfur but no significant amount of its carbon had been derived from the methionine. Thus, it has been established that the carbon chain of methionine is not utilized in the *in vivo* conversion of methionine to cystine.

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BIBLIOGRAPHY

1. Tarver, H., and Schmidt, C. L. A , J. Biol. Chem., **130,** 67 (1939).
2. Nicolet, B. H., J. Washington Acad. Sc., **28,** 84 (1938)
3. Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., Proc. Soc. Exp Biol. and Med., **35,** 501 (1936).
4. Toennies, G., J. Biol. Chem., **132,** 455 (1940).
5. du Vigneaud, V , Dyer, H. M., and Kies, M. W , J. Biol. Chem , **130,** 325 (1939).
6. Patterson, W. I , and du Vigneaud, V., J. Biol. Chem., **123,** 327 (1938).
7. du Vigneaud, V , Brown, G. B., and Chandler, J. P , J Biol. Chem , **143, 59** (1942).
8. Binkley, F , Anslow, W. P , Jr., and du Vigneaud, V., J. Biol Chem., **143, 559** (1942)
9 Binkley, F., and du Vigneaud, V , J. Biol. Chem., **144,** 507 (1942)
10. Stetten, D , Jr , J Biol. Chem., **144,** 501 (1942).
11. Kilmer, G W , and du Vigneaud, V., J. Biol Chem., **154,** 247 (1944).
12. Rose, W C , and Rice, E. E., J. Biol. Chem , **130,** 305 (1939).
13. du Vigneaud, V., Chandler, J P , Moyer, A W., and Keppel, D. M., J. Biol. Chem , **131,** 57 (1939).
14. Osborne, T B , and Mendel, L. B., J Biol. Chem., **37,** 572 (1919).
15. Graff, S , Maculla, E , and Graff, A. M., J. Biol. Chem., **121,** 81 (1937).
16. Heard, E V , and Lewis, H. B., J. Biol. Chem., **123,** 203 (1938).
17. Dawbarn, M , Australian J. Exp Biol. and Med. Sc , **16,** 159 (1938); Chem Abstr , **32,** 8490 (1938)
18. Rittenberg, D , Keston, A. S., Rosebury, F., and Schoenheimer, R , J. Biol. Chem , **127,** 291 (1939).

THE DETERMINATION OF BLOOD PLASMA IRON*

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Hematopoiesis has become extremely important because of the increased phlebotomy in obtaining blood plasma. Many studies have been made to show the relationship of nutrition to the rate of blood regeneration. During the course of an investigation with experimental anemia and the utilization of iron in foods, a study of the relationship of the plasma iron to the regeneration of hemoglobin was begun. To do this, there was required a rapid and accurate method for the determination of the plasma iron in numerous samples of blood.

Many methods have been published, all of them based on the precipitation or digestion of the plasma proteins and the final colorimetric measurement of the iron. Fowweather (1) found that the precipitation of the proteins with trichloroacetic acid gave lower values than total digestion of the plasma with sulfuric acid and hydrogen peroxide. Because traces of hemoglobin gave high values when the total digestion method was used, he analyzed only samples of plasma that gave a negative test with benzidine. Both Barkan (2, 3) and Tompsett (4) have studied the use of trichloroacetic acid to precipitate the plasma proteins. Barkan found that incubation of the plasma at 37° in the presence of 1.2 per cent hydrochloric acid prevented the coprecipitation of the inorganic iron and gave good recoveries of added iron. Tompsett maintained that ferric iron forms a protein complex more readily than iron in the ferrous state. He found that reduction of the ferric iron with thiolacetic acid or sodium hydrosulfite or the formation of the ferric pyrophosphate complex prevented the coprecipitation of the iron by the proteins. Shorland and Wall (5) and Borgen and Elvehjem (6) found that thiolacetic acid and sodium hydrosulfite reacted with traces of hematin and hemoglobin, releasing iron to give abnormally high values. They found that sodium pyrophosphate gave low values when added to the sample of blood unless a reducing agent was also present. Because of these conflicting reports and because Barkan's procedure (3) is not convenient, Schaefer and McKibbin[1] of this laboratory investigated the problem and devised a method for the determination of the plasma iron that is simple

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[1] Schaefer, A., and McKibbin, J., unpublished report.

and seemed to give accurate results. Their method was based on the observation that the inorganic iron was distributed in equal concentration between the filtrate and the precipitate when the proteins were precipitated with trichloroacetic acid. This method has been used in previous investigations and the values obtained agree very well with those reported by others. When this new problem was undertaken, this same method was used but it was found that consistent results were not obtained. A need for a method that would give accurate and reproducible values was apparent.

Reagents—

1. Trichloroacetic acid, 25 per cent solution in water. The trichloroacetic acid should be redistilled if it is not free of iron.

2. Hydrochloric acid, 6 N, redistilled.

3. Ammonium hydroxide, approximately 6 N.

4. Buffer solution, pH 4.58, 27.2 ml. of C.P. glacial acetic acid and 33.4 gm. of C.P. sodium acetate (anhydrous) dissolved in water and made to a volume of 250 ml.

5. Thioglycolic acid, Eastman's practical grade.

6. *p*-Nitrophenol, 0.1 per cent solution in water.

7. α, α'-Bipyridine, 0.2 per cent solution in 5 per cent acetic acid. Dissolve 0.2 gm. of the reagent in 5 ml. of C.P. glacial acetic acid and dilute to 100 ml. with water.

8. Standard iron solution. Dissolve iron wire (99.8 per cent) in a mixture of nitric acid and hydrochloric acid and dilute with water to give a concentration of 1 mg. per ml. From this stock solution standard solutions of 100 and 1 γ per ml. are made.

9. Iron-free water. Distilled water should be redistilled from glass. All equipment should be thoroughly cleaned and rinsed with iron-free water.

EXPERIMENTAL

In order to use the minimum amount of plasma and yet obtain enough iron for the formation of sufficient color intensity, α, α'-bipyridine was selected as the reagent. α, α'-Bipyridine forms an intense pink color with ferrous iron which has an absorption maximum at about 520 mμ and can be used to detect less than 0.05 part per million. Since the iron must be in the ferrous state, thioglycolic acid was used as the reducing agent. It is a very strong reducing agent and only 2 drops of the undiluted reagent are required. Although variations in pH between 3 and 9 do not influence the intensity or hue of the color produced with bipyridine (7), a constant pH was maintained in the standard and test solutions. An acetic acid-acetate buffer was chosen which buffered at pH 4.6. At this pH the color formation is almost instantaneous.

It was found that because of the small amounts of inorganic iron in blood

plasma it would be necessary to measure the color intensity obtained with 1 to 4 γ of iron. The sensitivity of the test for iron with bipyridine was determined by measuring the color obtained with 0.5, 1.0, 2.0, 3.0, and 4.0 γ of iron in 11 ml. of solution. The Evelyn colorimeter was used to measure the intensity of the color. Filter 520 was used, and the transmission of light was read on the galvanometer scale to the nearest 0.1 of a scale division. The L values ($L = 2 - \log G$) were calculated from galvanometer readings (G) and plotted against micrograms of iron (Fig. 1). After a series of similar experiments, it was found that 0.5 to 1 γ of iron can be determined with an accuracy of ± 6 per cent. The accuracy for 1 to 2 γ is ± 3 per cent, and ± 2 per cent for 2 to 4 γ.

Various recovery experiments were conducted by the Schaefer-McKibbin[1] method. The procedure is as follows: 5 ml. of blood plasma which had
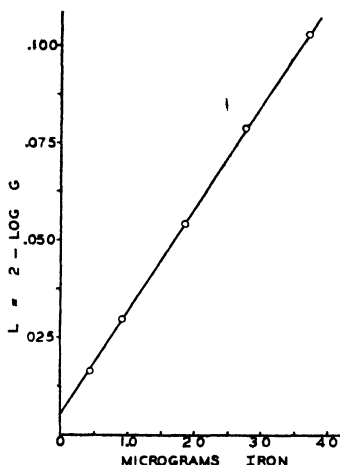
FIG. 1. Standard iron curve

been obtained from a dog by venipuncture, a small quantity of a saturated sodium oxalate solution being used to prevent clotting, were pipetted into a 15 ml. calibrated centrifuge tube. 5 ml. of 10 per cent trichloroacetic acid solution were added and mixed with the plasma. If a recovery experiment were to be made, the iron solution containing a known amount of iron, e.g. 2, 4, 6, 8, and 10 γ, was added before the trichloroacetic acid was mixed with the blood plasma. The tubes were then placed in a water bath (80–90°) for 5 minutes, and then centrifuged for 15 minutes at 2000 R.P.M. The supernatant liquid was poured off into another 15 ml. calibrated centrifuge tube after the volume of the precipitate and of the clear solution had been noted. The iron was then determined in the clear filtrate or an aliquot of

it. The results are tabulated in Table I. It is seen that the amount of added iron recovered in the filtrate varied from 50 to 78 per cent. If the amount found in the precipitate was also included, the total recovery ranged from 83 to 108 per cent. This was an indication that iron was carried down with the precipitate and that the relationship between the iron in the filtrate and the total iron was variable. An attempt was made to wash the precipitate with a mixture of water and trichloroacetic acid but the recoveries were not appreciably improved. It was necessary to devise a method to eliminate the "carry down" of the iron with the precipitate, or one which would permit the recovery of all the iron by the simple procedure of washing the precipitate.

Since Tompsett (4) found that thioglycolic acid and sodium hydrosulfite improved the recoveries in his method, it was decided to test the effect of reducing agents on the iron content of plasma and on the recovery of added

TABLE I

Experiments on Recovery of Iron from 5 Ml. of Plasma by Schaefer-McKibbin Method

Iron added	Added iron in filtrate		Iron recovered	
γ	*γ*	*per cent*	*γ*	*per cent*
2.00	1.38	69.0	1.86	93.0
4.00	2.62	65.5	3.84	96.0
4.00	3.12	78.0	4.33	108
6.00	3.52	58.7	4.98	83.0
6.00	3.57	59.5	5.23	87.0
8.00	5.04	63.0	7.23	90.4
10.0	5.05	50.5	8.63	86.3

iron. The blood plasma used in this experiment was obtained from dogs. Since the blood had been allowed to remain in the ice chest for 12 hours before centrifugation, there was quite a bit of hemoglobin in the plasma. The Schaefer-McKibbin method[1] was followed and the reducing agent was added to the plasma before the proteins were precipitated. The precipitate was washed with a mixture of the trichloroacetic acid solution and water, and this was added to the filtrate. The results are tabulated in Table II. It will be noted that the plasma iron in Groups II, III, IV, and V is very high when compared to the other groups. It is an indication that iron other than the plasma iron was being measured. This is similar to the results of Shorland and Wall (5), and Borgen and Elvehjem (6). They found that the non-hemoglobin iron content of blood is increased when thioglycolic acid or sodium hydrosulfite is added before the proteins are precipitated.

In order to obviate the situation that was encountered in the preceding experiment, fresh plasma, free of hemoglobin except perhaps for traces

which could not be detected, was used. The experiment was repeated, and now the abnormally high values were not obtained with the strong reducing agents, but the recovery of added iron was not good in all cases. The main difficulty seemed to be the coprecipitation of the iron and the incomplete removal of this iron by washing. It is believed that the iron is mechanically carried down by the precipitate. It had been noted that the precipitate formed by the addition of trichloroacetic acid to the plasma was very fine and seemed to be quite hydrated, settling to the bottom of the centrifuge tube to form a thick mass. The problem seemed to be 2-fold: first, to reduce any plasma iron that may be in the ferric state so that protein complexes are not formed, and second, to precipitate the proteins so that very

TABLE II

Effect of Reducing Agents

Group No.	Additions to 5 ml plasma	Iron added	Iron in 5 ml. plasma	Iron recovered
		γ	γ	per cent
I	None	0	13.0	78.0
	"	8.32	19.5	
II	5 drops thioglycolic acid	0	33.0	162
	5 " " "	8.32	46.5	
III	10 " " "	0	61.7	110
	10 " " "	8.32	71.0	
IV	2 ml. 4% $Na_2S_2O_4$	0	32.0	119
	2 " 4% "	8.32	42.0	
V	2 " 10% "	0	37.0	134
	2 " 10% "	8.32	48.3	
VI	1 " 10% $NH_2OH\text{-}HCl$	0	12.5	90
	1 " 10% "	8.32	20.0	
VII	100 mg vitamin C	0	12.2	

little iron is carried down. The solution to both of these problems came when the properties of the proteins in question were taken into account. Mirsky and Anson (8) maintain that denaturation of most proteins releases sulfhydryl groups which have reducing properties. They recognize also the presence in proteins of non-sulfhydryl reducing groups which they ascribe to the tyrosine and tryptophane residue. They found that denatured serum globin would reduce the ferricyanide ion. Jaques (9) found that fibrinogen can reduce dilute solutions of iodine and of hydrogen peroxide rapidly at 25° at pH 5 to 7. The reducing power of fibrinogen is not changed by denaturation, but the reducing power toward hydrogen peroxide is increased on conversion to fibrin. He also noted that denaturation of the serum proteins at 100° increased the reduction of iodine by 20 per cent. In

following these leads, we found that when the plasma is heated at 100° a gel is formed. Upon addition of trichloroacetic acid a light, curdy precipitate results, the very nature of which facilitates the removal of any residue iron. The following procedure has been perfected so that reproducible and consistent results were obtained with good recoveries of added iron. This method does not require blood plasma free of hemoglobin because any hemoglobin is precipitated by the trichloroacetic acid.

Proposed Procedure

3 to 5 ml. of blood plasma containing about 3 to 9 γ of inorganic iron are pipetted into an ungraduated Pyrex 15 ml. conical centrifuge tube. 3 ml. of redistilled water are added and mixed with the plasma. A blank consisting of all the reagents used is also prepared.

Place the tubes in boiling water for 2 to 3 minutes, or until the solution becomes opaque. Cool the tubes in cold water.

Add 2 ml. of 25 per cent trichloroacetic acid solution, and stir thoroughly so that the acid is intimately mixed with the plasma. Use a small blunt end stirring rod so as not to break through the bottom of the tubes.

Place the tubes in a water bath at 90–95° for 3 minutes, stirring the solution once or twice.

Remove from the bath and cool in cold water. Centrifuge at 2000 to 3000 R.P.M. for 5 minutes.

Decant the supernatant liquid into a 15 ml graduated centrifuge tube.

Add 4 ml of redistilled water and 1 ml. of trichloroacetic acid to the original tube. Break up the precipitate and stir well. Place in a water bath at 90–95° for 3 minutes, stirring each solution once. Remove and cool the tubes in cold water.

Centrifuge the tubes for 5 minutes at 2000 to 3000 R.P.M. and decant the supernatant liquid into the tube which contains the first filtrate.

Add 1 drop of 0.1 per cent p-nitrophenol indicator solution, and add NH$_4$OH drop by drop until the solution becomes yellow.

Add 1 ml. of the buffer solution and enough water to make 15 ml. Mix.

Pipette an aliquot, 5 or 10 ml., containing 2 to 3 γ of iron into an Evelyn tube, and add 2 drops of thioglycolic acid and mix. If less than 10 ml. are taken, add water to make 10 ml.

Determine the center setting (100 per cent transmission on the galvanometer scale) for each solution in the colorimeter.

Add 1 ml. of 0.2 per cent α, α'-bipyridine reagent and mix by gentle shaking or tapping of the tube. Read each tube in the colorimeter with the respective center setting previously determined. The galvanometer (G) reading is recorded to the nearest 0.1 of a scale division if the Evelyn colorimeter is used.

Calculate the amount of iron in the solution in each tube by reference to a standard curve or use the following formula after calculating the L values from the G readings.

$$\frac{\text{Micrograms iron}}{\text{Aliquot}} = 40.6 \times L_{\text{aliquot}} - L_{\text{reagent blank}}$$

In order to test the validity of the proposed method, recovery experiments were made which are summarized in Table III. The recovery of added iron ranges from 92 to 104 per cent. Considering the sensitivity of

TABLE III

Recovery of Added Iron

Dog No.	Sample	Iron added	Iron found	Iron recovered
	ml.	γ	γ	*per cent*
335	5	3.12	2.94	94.5
340	3	1.04	0.96	92.3
338	5	4.16	4.00	96.0
325	3	1.04	1.05	101
337	5	2.08	2.16	104

TABLE IV

Plasma Iron Values of Normal and Anemic Dogs

Dog No	Age	Ration	Iron in 100 ml plasma
	mos.		γ
333	1	Synthetic ration	90.0
334	1	Mineralized milk + vitamins	102
335	1	" " + " without added iron	32.0
325	9	Synthetic ration	200
300	12	Mineralized milk + vitamins	141

the method and the error incurred by the use of the Evelyn colorimeter with colored solutions of low intensity, these recoveries are good. Recovery of the added constituent does not entirely determine the precision of a method. Reproducibility of results is also important, and we have found that samples of the same blood gave remarkably similar results.

This proposed method was applied by Messrs. Michaud and Ruegamer to their work with nutritional anemia. In numerous analyses of blood plasma, they obtained reproducible values and consistent results. Some typical data as found by them are listed in Table IV.

SUMMARY

1. A method has been devised to measure the plasma iron. The difficulties encountered because of the precipitation of the plasma proteins by trichloroacetic acid were obviated by a simple procedure. The proteins are denatured by heat before being precipitated by the trichloroacetic acid. One washing of the precipitate seems to recover all the iron.

2. α, α'-Bipyridine was used to measure the iron colorimetrically in the range of 1 to 4 γ. When an acetic acid-acetate buffer at pH 4.6 is used, the color formation is instantaneous.

3. The plasma iron may be determined with a maximum error of ± 10 per cent for blood plasma containing 30 to 90 γ per cent of iron, and with a maximum error of ± 5 per cent for blood plasma containing 100 to 200 γ per cent of iron.

BIBLIOGRAPHY

1. Fowweather, F. S., *Biochem. J.*, **28**, 1160 (1934).
2. Barkan, G., *Z. physiol. Chem* , **148**, 124 (1925).
3. Barkan, G., and Walker, B. S , *J. Biol. Chem.*, **135**, 37 (1940)
4. Tompsett, S. L , *Biochem J* , **28**, 959, 1536 (1934).
5. Shorland, F B., and Wall, E M., *Biochem J.*, **30**, 1049 (1936).
6. Borgen, D R , and Elvehjem, C. A , *J. Biol. Chem.*, **119**, 725 (1937).
7. Moss, M. L., and Mellon, M G , *Ind and Eng. Chem* , *Anal. Ed.*, **14**, 862 (1942).
8. Mirsky, A. E , and Anson, M L , *J Gen. Physiol.*, **19**, 451 (1936).
9. Jaques, L. B , *Biochem. J.*, **37**, 344 (1943).

FERRITIN

VIII. SPEED OF UPTAKE OF IRON BY THE LIVER AND ITS CONVERSION TO FERRITIN IRON

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In a previous paper (1) it was demonstrated by means of radioactive iron as a tracer that 80 per cent of the ferric ammonium citrate iron some 13 days after intravenous injection was found in the liver. Of the crystalline ferritin isolated from this liver, three-fourths of the iron in the ferritin had come from the injected iron. In the present paper we have studied the composition of the iron complex in ferritin, the mechanism and location of the conversion of inorganic to ferritin iron, and the speed with which this conversion occurs.

Composition of Iron Micelle in Ferritin—From ultracentrifuge studies (2) it has been shown that crystalline ferritin is made up of about 20 per cent of apoferritin molecules free from iron, the remaining 80 per cent of apoferritin having the iron micelles firmly bound to some portions of the protein surfaces. The iron in ferritin is characterized by its unique magnetic susceptibility (3), revealing that there are 3 unpaired electrons per iron atom. This property readily distinguishes it from all other iron compounds known to occur normally in the organism.

When ferritin is treated with 1 N NaOH for 10 minutes at room temperature, the protein is denatured, remaining in solution, and a red-brown coagulum separates out which contains all of the iron. The brown substance was centrifuged down, dialyzed, and dried over P_2O_5 to constant weight. The analysis of this material is given in Column 1 of Table I. In Column 4 the composition of the ash has been calculated and agrees with the experimentally found value. In calculation of the composition of the iron micelles (Columns 2 and 3) it has been assumed that the empirical formula of the iron is FeOOH, with all of the phosphate in salt linkage with the iron. It has also been assumed that the nitrogen represents protein occluded on the coagulated iron micelles. The formula FeOOH corresponds to the composition of the naturally occurring minerals, goethite and lepidocrocite. However, in these minerals the magnetic susceptibility per gm. atom of Fe, measured at room temperature, is much lower than in ferritin.

The composition of the alkali-treated micelle is not that of the intact

micelle in ferritin. It has been shown that all the phosphorus of ferritin is bound to the iron micelles and is not a constituent of the apoferritin. In the intact micelle, the ratio of phosphorus to iron is about 1 P atom to 8 to 9 Fe atoms. On treatment with alkali, approximately 75 per cent of this phosphorus is removed, so that a chemical change in the micelles is recognizable (4). However, the magnetic measurements on the alkali-treated material show that the type of bonding for the iron atom as manifested in its magnetic susceptibility has remained unchanged. The ortho-phosphoric acid groups were evidently hydrolyzed off by alkali treatment, the phosphate group being replaced by an OH without changing the type of bonding to the Fe atom.

TABLE I

Per Cent Composition of Iron Micelles, Separated from Ferritin by Treatment with Alkali

Analysis of iron micelle		Composition based on Fe as FeOOH				Composition of ash*	
(1)		(2)		(3)		(4)	
C	6.79	FeOOH	82.8	FeOOH	82.8	1.31% Fe in $FePO_4$	3.6
H	1.61	$FeOPO_3H_2$	4.77	$FeOPO_3H_2$	4.77	52.1% Fe in Fe_2O_3	74.5
N	1.65	CdO	1.98	CdO	1.98	CdO	1.98
P	0.73	Protein	10.3	N	1.65	Calculated	80.08
S	0.05	(calculated		H	1.61	Found	80.93
Fe	52.7,	from N)		C	6 79		
	53.8	Total	99.8		99.6		
Cd	1.75			O†	2		
Ash 80.93				Total	101.6		

Magnetic susceptibility corresponds to an effective magnetic moment of 3.8 Bohr magnetons per gm. atom of iron.

* Assume that all of the phosphate is in the form of $FePO_4$
† Oxygen correction calculated from O:N ratio in protein.

The density of the intact iron micelles may be calculated from the values of Rothen (2) for the density of apoferritin (1.34) and of ferritin containing 23 per cent iron (1.75). If one assumes the iron to be in the form of $(FeOOH)_8(FeO—OPO_3H_2)$, then the micelles make up 38 per cent by weight of the ferritin and the density of the micelles is 3.50 gm. per cc. On the other hand, if one assumes the iron to be merely in the form of FeOOH, the density of the micelles is 3.83. The density of minerals with the composition FeOOH is 4.2 ± 0.2. The density of Fe_2O_3 minerals is about 5.0. Assuming micelles with relatively large surface and looser packing than in the crystalline minerals, owing to the presence of phosphate, the density of ferritin micelles as 3.50 is a reasonable figure, supporting the formula $(FeOOH)_8 (FeO—OPO_3H_2)$.

The question always arises in dealing with micelles of large surface as to whether the phosphate may merely be adsorbed to the surface of the iron micelles, or more firmly bound. That only a small portion of the phosphate may be loosely held by adsorption to the iron micelles is suggested by the following experiment. Solutions of ferritin and of apoferritin were mixed with radioactive phosphate at pH 7. After 30 minutes the proteins were crystallized out of solution with the aid of $CdSO_4$, the crystals were centrifuged, washed, dissolved in 5 per cent ammonium sulfate solution, and the solution was analyzed for radioactive PO_4. From Table II it is seen that only negligible amounts of PO_4 were adsorbed. The adsorption by ferritin of radioactive PO_4 amounted to only 4 per cent of the PO_4 originally present in this ferritin.

In order to determine whether the phosphorus of the micelle is localized on the surface or is distributed throughout the-micelle, it would be necessary to remove the phosphate without destroying the ferritin. This can be accomplished with ammonium magnesium citrate, by taking advantage

TABLE II

Adsorption of Radioactive Phosphate from Solution by Ferritin and Apoferritin

	Radioactive PO_4, 1 cc., pH 7	Radioactive P found in crystalline material	Radioactive P adsorbed
	mg P	*mg*	*per cent*
5 cc. apoferritin (50 mg. dry weight)	0.1	0.001	1
5 " ferritin (50 mg. dry weight)	0.1	0.004	4

of the great stability of apoferritin in dilute ammoniacal solutions. 10 cc. of ferritin (120 mg. dry weight) were treated with 2 cc. of 0.5 M ammonium magnesium citrate. After several days, the crystals of ammonium magnesium phosphate which had formed were centrifuged down, the clear solution was dialyzed free from NH_4OH, and then the ferritin was crystallized out with $CdSO_4$. Analyses for Fe and P were made on these crystals. Although the iron decreased, it is seen from Table III that the phosphorus decreased more rapidly than the iron. If PO_4 groups were present only on the surface of the micelles, the tendency should be to reach values for a ferritin devoid of phosphorus. The ratio of Fe:P did decrease but not sufficiently, and it seems reasonable to interpret the data as indicating that PO_4 is present both on the surface and also bound within the micelle.

Conversion in Vitro of Ferric Ammonium Citrate (FAC) to Ferritin Iron—Ferric iron may exist in three paramagnetic states; namely, those with 1, 3, or 5 unpaired electrons. It was shown (3) that for even a simple

substance like ferric chloride, depending on its method of hydrolytic treatment, one could obtain mixtures of these states varying from 1 up to 5 unpaired electrons, but never a pure 3 unpaired electron state. It is possible that these intermediate values represented mixtures of the 1 and 5 electron states. Since FAC could be injected into the blood stream and recovered from the liver as ferritin iron, it becomes necessary to determine whether this conversion can occur in the blood stream or only in the liver cells, and whether the conversion is an enzymatically controlled process.

Depending on the method of preparation the magnetic susceptibility of green FAC is found to vary. Belloni (5) reports a compound of 3 Fe to 2 citric acid molecules. Preparation A (Table IV) was made on this

TABLE III

Removal of Phosphorus from Micelles with Ammonium Magnesium Citrate

Days of treatment	Per cent Fe	Per cent P	No of Fe atoms to 1 P atom
0	17.9	1.27	7.8
2	14.8	0.76	10.8
5	11.9	0.51	12.9

TABLE IV

Magnetic Moments of Some Ferric Ammonium Citrate Preparations

Preparation	Composition of ferric ammonium citrate	pH	Bohr magnetons
A	Fe to citrate = 3:2	7.06	4.67
B	FAC in slight excess of ammonium citrate	7 73	5.69
C	Commercial preparation	6.03	4.67

basis by mixing 5 cc. of 1.065 M $FeCl_3 \cdot 6H_2O$ with 5.66 cc. of 1.46 M citric acid, adding 3 cc. of concentrated NH_4OH, and diluting to 25 cc. Preparation B was the radioactive material used for the conversion experiments in the dogs. It was made by dissolving radioactive ferric hydroxide in a slight excess of citric acid, adding concentrated NH_4OH, and removing the excess NH_4OH on a steam bath; this preparation therefore contains an excess of ammonium citrate. Preparation C was a commercial green iron citrate (Lilly) used for parenteral injection.

In order to determine whether FAC can undergo a change in paramagnetism by mere contact with blood plasma, the FAC (Preparation A of Table IV) was mixed with dog plasma, allowed to stand for half an hour at 38°, and then measured magnetically. Table V shows the results of

this experiment and indicates that there is no appreciable change in magnetic susceptibility of FAC when mixed with serum, and a possible slight diminution when diluted with water.

The possibility suggested itself that the ferritin micelles might increase in size by a kind of crystal growth in the presence of either ferrous or ferric iron. At first this appeared likely from the data of the following experiment. 5 cc. of protein (either apoferritin or ferritin) were mixed with 0.5 cc. of radioactive FAC, green-brown in color ($\approx$0.453 mg. of Fe), or this FAC was reduced to the ferrous form with hydrosulfite after the mixing. After the mixture stood overnight at room temperature, CdSO$_4$

TABLE V

Paramagnetism of Ferric Ammonium Citrate in Serum

Solutions	pH	Bohr magnetons
10 cc. dog plasma + 0.5 cc. H$_2$O..	7.82	
10 " H$_2$O + 0.5 cc. FAC, Preparation A..	7.38	4.43
10 " dog plasma + 0.5 cc. FAC, Preparation A. ..	7.68	4.52

TABLE VI

Radioactive Iron Uptake from Ferric Ammonium Citrate by Protein

Protein material	Level of oxidation of iron	Radioactive iron taken up by	
		Once crystallized material	Twice crystallized material
		per cent	per cent
Apoferritin (55 mg.)	Fe^{+++}	9.0	1.1
" (55 ")	Fe^{++*}	2.0	
Ferritin (82 mg.) . .	Fe^{+++}	47.7	42.0
" (82 ")	Fe^{++*}	31.3	

* Fe^{++} represents ferrous ammonium citrate produced by adding 5 mg. of sodium hydrosulfite to the mixture.

was added, and the crystals were isolated, washed three times with 5 per cent CdSO$_4$ to remove any adhering FAC, and the radioactivity measured in order to determine how much iron had been taken up. Although 42 per cent of the FAC is taken up by ferritin (Table VI), actually this represents only 0.19/14.8 $\times$ 100 = 1.28 per cent increase in iron over that originally present in ferritin. Because of the small increase in magnetic susceptibility, magnetic measurements could not indicate whether the micelles were able to orient the newly acquired iron in their own unique pattern.

The following experiment, however, indicates that what is actually

happening is not a micellar growth characteristic of ferritin but rather that colloidal micelles of brown or yellow ferric hydroxide, originating in the FAC, become adsorbed to the ferritin micelles. A freshly prepared green FAC similar to Preparation A, Table IV, was added to ferritin, which was then crystallized. It took on about 2.5 per cent of the radioactive iron added. When this FAC was treated with NH_4OH to bring the pH to 10, and the material then heated to remove excess NH_4OH, a brownish yellow form of FAC appeared. The ferritin adsorbed this material to the extent of 8 per cent. If ferritin is heated to 80° in the presence of physiological saline and freshly prepared green FAC, the ferritin will adsorb about 10 per cent of the radioactive iron added. It should be pointed out here that if this accretion of iron micelles onto ferritin were the normal process in the liver, the ferritin isolated from the liver would not be expected to possess its characteristic constant value for magnetic susceptibility.

The following experiments *in vitro* suggest that the synthesis of ferritin is specifically catalyzed, although the extent of synthesis under these conditions was very low (around 0.2 per cent of the radioactive iron being incorporated into the isolated ferritin). 70 gm. of fresh weight of guinea pig liver kept in the ice box overnight were ground in a blendor with 50 cc. of isotonic saline + 6 cc. of horse apoferritin[1] (66 mg. dry weight). The suspension was divided into two equal portions of 60 cc. each. To Portion A was added 1 cc. of radioactive FAC (≈ 0.45 mg. of Fe). This was stoppered, placed at 40° for 1 hour to facilitate reduction, and then shaken with air at 30° for 1 hour. It was then heated to 80° and treated by the usual method for ferritin isolation as the crystalline material. A pale colored ferritin resulted, the packed crystals having a volume of 0.073 cc. and having a radioactivity of 1438 counts per minute. The control, Portion B, was treated in the same manner as Portion A, except that the radioactive FAC was added to the liver suspension just before it was heated to 80°. The crystalline material isolated from the control had a volume of 0.070 cc. and a radioactivity of 908 counts per minute. The radioactivity of Portion A is therefore about 58 per cent higher than that of the control Portion B.

In another experiment 80 gm. of fresh guinea pig liver were treated with 70 cc. of isotonic saline + 6 cc. of apoferritin. The suspension was divided into two portions of 80 cc. each. To Portion A was added 0.5

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[1] The horse apoferritin was added so that a crystalline material could readily be obtained from guinea pig liver, the size and shape of the protein of guinea pig and horse livers being such that they crystallize together. Thus even small amounts of ferritin molecules from guinea pigs could be handled. In addition, the horse apoferritin even in the presence of enzymes from the liver of guinea pigs might function to acquire ferritin iron micelles.

cc. of radioactive FAC ($\backsim$0.225 mg. of Fe). It was stoppered, placed at 40° for 1 hour, shaken with air at 35° for the next hour, and, just before it was heated to 80°, 0.5 cc. of non-radioactive FAC ($\backsim$0.25 mg. of Fe) was added. The volume of crystalline ferritin isolated was 0.12 cc. and its radioactivity was 1088 counts per minute. The control Portion B was treated in the following manner. To 10 cc. of the suspension was added 0.5 cc. of non-radioactive FAC ($\backsim$0.25 mg. of Fe); the mixture was placed at 40° for 1 hour, shaken with air at 35° for the next hour, and, just before it was heated to 80°, 0.5 cc. of radioactive FAC ($\backsim$0.225 mg. of Fe) was added. The volume of crystalline ferritin isolated was 0.14 cc. and its radioactivity was 790 counts per minute. The radioactivity of Portion A is therefore 38 per cent higher than that of control Portion B.

Conversion in Vivo of FAC to Ferritin Iron—It had previously been shown (1) that 13 days after radioactive FAC was injected intravenously into a dog 82 per cent of the radioactive iron could be found in the liver. Of the crystalline ferritin isolated from the dog liver, about 75 per cent of the iron was represented by the radioactive iron injected. The ferritin iron was shown to possess the magnetic moment typical of the normal ferritin iron; namely, 3.8 Bohr magnetons per gm. atom of iron.

The following experiments were undertaken to determine how rapidly this conversion of injected iron into ferritin iron could occur. Each of three dogs was injected intravenously with radioactive FAC ($\backsim$9.2 mg. of Fe) having a magnetic moment of 5.69 Bohr magnetons per gm. atom of iron. The dogs were sacrificed after 1, 2, and 5 hours. The radioactivity of the spleens and livers was determined, the brown solutions of ferritin + non-crystallizable ferritin (F + NCF) and the crystalline material designated as ferritin were isolated and their radioactivity also determined. The various techniques of measurement have already been described (1). The magnetic measurements were supervised by Dr. L. Michaelis.

The speed with which FAC is taken up by the liver from the blood stream is evident from Table VII. 1 hour after injection, the liver has already taken up over 40 per cent of the total injected iron. The uptake of iron by the spleen is seen to be negligible. The F + NCF fractions of the 1 and 2 hour experiments appear to have a higher specific radioactivity than the iron of whole liver, indicating a preferential conversion of newly incoming iron into ferritin iron. The red-brown color and the magnetic moments of these 1 and 2 hour F + NCF fractions are typical of pure ferritin solutions.

However, the 5 hour experiment represents a striking deviation from the expected results. Here, the liver has accumulated radioactive iron to the extent of 40 per cent of the iron injected; yet this radioactive iron

has not been converted to ferritin iron since the F + NCF fraction was of a brown-yellow color and the magnetic moment was 4.72 Bohr magnetons, a value which is much higher than for normal ferritin (*i.e.* 3.8 Bohr magnetons). No crystallizable ferritin could be obtained from this F + NCF fraction. At the same time the spleen of this animal had been noted to be abnormally swollen and congested. This abnormal behavior suggests that the liver could pick up the iron circulating in the blood stream but

TABLE VII

Conversion of Ferric Ammonium Citrate into Ferritin Iron

Time after injection		Total Fe	Total activity	Specific activity	Distribution of labeled Fe as per cent of injected Fe	Color	Magnetic moment
hrs.		*mg*	*counts per min*	*counts per min per mg. Fe*			*Bohr magnetons per gm atom Fe*
	Injected radioactive ferric ammonium citrate by vein	9.2	274,000	29,800	100		
1	Liver (178.5 gm. fresh weight)	80.4	113,000	1,405	41.3		
	Liver F + NCF	24.5	54,300	2,210	19.8	Red-brown	3.78
	" ferritin	5.48	12,100	2,210	4.4		
	Spleen (9 gm. fresh weight)		166		0.06		
2	Liver (255 gm. fresh weight)	118	167,000	1,410	61.0		
	Liver F + NCF	20.2	59,600	2,950	21.8	Red-brown	3.80
	" ferritin	5 56	18,300	3,290	6.7		
	Spleen (11.5 gm. fresh weight)		690		0.25		
5	Liver (377 gm. fresh weight)	108	115,000	1,030	40.7		
	Liver F + NCF	9.00	51,600	5,730	18.8	Brown-yellow	4.72

that the enzymatic mechanism for converting it to ferritin iron was deranged. In this connection it may be mentioned that the great variation in ferritin content found in human livers (6) and the occasional very low values suggest that derangement of ferritin synthesis may not be of uncommon occurrence in man.

We wish to acknowledge our gratitude to Dr. L. Michaelis for his advice and interest in these studies.

SUMMARY

The approximate composition of the iron-rich micelles of ferritin appears to be $(FeOOH)_8 (FeO—OPO_3H_2)$. This structure is supported by analytical data for the micellar brown material split off from the protein with alkali, and the identity of the paramagnetic susceptibility of this material with that of intact ferritin. The negligible tendency for ferritin to adsorb radioactive phosphate, and the difficulty of removing all of the phosphate from intact ferritin by means of ammonium magnesium citrate, suggest that the phosphate groups are distributed throughout the micelles, probably in linkage to the iron, this linkage being hydrolyzed by dilute alkali at room temperature. The density of the intact micelles also lends support to this formula.

The *in vitro* conversion of radioactive ferric ammonium citrate (FAC) to ferritin has been studied. The paramagnetism of FAC itself depends on its method of preparation. Its paramagnetism is not significantly changed when mixed with dog serum. Ferritin may take up almost 50 per cent of radioactive iron by mixture with a preparation of radioactive FAC. This is shown not to be a specific crystalline growth of ferritin micelles but rather an adsorption of ferric hydroxide micelles already present in the FAC. The incubation of guinea pig liver brei with apoferritin and radioactive FAC, the subsequent isolation of crystalline ferritin, and the determination of its radioactivity indicate that iron micelles of ferritin are the result of metabolic synthesis, perhaps due to a specific enzyme.

By injecting radioactive FAC intravenously into dogs and isolating the ferritin-rich fraction from liver (F + NCF), it has been found that 1 hour after injection over 40 per cent of the injected iron was present in the liver. In another dog, after 2 hours, 61 per cent of the injected iron was present in the liver. The ferritin-rich fraction of these dogs is of normal brown color and gives the characteristic value for the paramagnetism of ferritin. However, in a third dog sacrificed after 5 hours, although the liver had taken up 40 per cent of the injected iron, the iron-rich fraction as isolated was pale brownish yellow in color with a paramagnetism of 4.7 Bohr magnetons, indicating a derangement of the mechanism for ferritin synthesis.

BIBLIOGRAPHY

1. Hahn, P. F., Granick, S., Bale, W. F., and Michaelis, L, *J. Biol Chem.*, **150,** 407 (1943)
2. Rothen, A., *J. Biol. Chem.*, **152,** 679 (1944).
3. Michaelis, L., Coryell, C. D, and Granick, S., *J. Biol. Chem.*, **148,** 463 (1943).
4. Granick, S., *J. Biol. Chem.*, **146,** 451 (1942).
5. Belloni, E., *Chem. Abstr.*, **15,** 839 (1921).
6. Granick, S., *J. Biol. Chem.*, **149,** 157 (1943).

ON THE QUANTITATIVE INCIDENCE OF CARBONIC ANHYDRASE IN THE CENTRAL NERVOUS SYSTEM

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With the Assistance of Dorothea V. Chan

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In a previous paper (1) data were presented indicating that in seven species of animals studied, including man, the carbonic anhydrase content of the spinal cord was approximately half that of the cerebrum. The data further indicated that this was not due to any differences in the relative amounts of the gray and white matter in the samples chosen, or to the enzyme accounted for by blood content.

The hypothesis was advanced that the greater enzyme content was associated with quantitative metabolic differences between the two areas which would play a part in establishing their respective functional levels. It was suggested that the enzyme was either in the neurons or in cells accessory to them, and that it was either produced *in situ* or was absorbed from the blood corpuscles which contain it in large amount. Differences in content in the latter event, perhaps due to differences in circulatory activity, would represent a result of function, but might in turn modify metabolism differentially.

Further data constituting an initial survey on the distribution of carbonic anhydrase in the central nervous system are presented below.

EXPERIMENTAL

Technique—The techniques used have been described (1, 2). In the study of the human brain two levels of white matter were established, one by removing a gyrus and separating the gray matter from the white. This white matter immediately below the cortex is designated "intragyral white matter." Specimens taken after removal of the gyrus are called "subgyral white matter" and represent fibers further removed from the cortex than the intragyral white matter. The human brain, Case A, was from a non-psychotic male; the others were from patients who died in a mental hospital.

Increase in Carbonic Anhydrase with Rostral Progression—Results from six species of animals are given in Fig. 1. The curve for the chicken represents the pooled material from five animals. In order to get enough material for two levels in the spinal cord of the guinea pig, three animals were used. Otherwise each curve records the data from one animal. The indices of content are placed centrally with reference to the general

area studied, the limitation of which was determined by the necessity of collecting approximately a gm. of material for the test. Data on the cerebellum are excluded and will be considered later. In thirteen curves representing five species, carbonic anhydrase increases rostrally.

Two curves on a steer and a calf are exceptions. In both the carbonic anhydrase content of the cerebrum was definitely lower than that of the
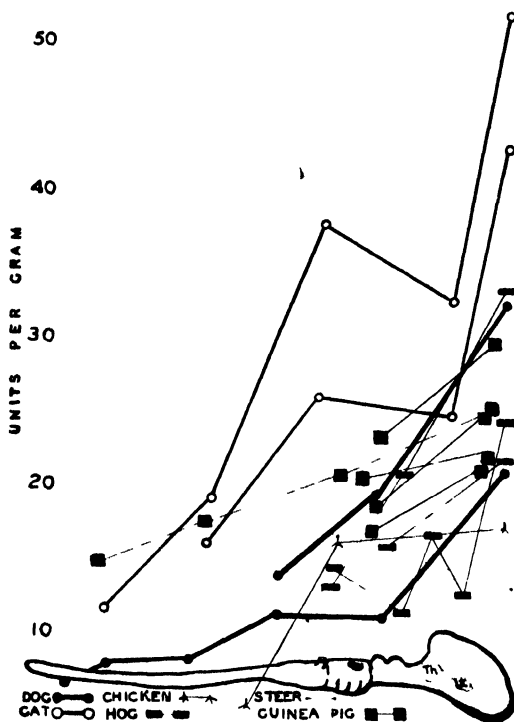
FIG. 1. Progression of carbonic anhydrase content through the cord to the cerebrum. Findings for the cerebellum are omitted. In the curve in which two sections from the cord of the guinea pig are given, the material from three animals was pooled. The curve for the chicken represents five animals pooled. The indices are placed approximately over the regions from which the samples were taken. *Th.* represents thalamus, *Lt.* lenticular nucleus.

brain stem. The content of the cerebellum was comparatively high in both animals. The large amount of material from the medulla oblongata of the steer was tested in some detail and is charted in Fig. 2 with the data for man.

Although in the cat, the dog, and the hog the carbonic anhydrase content of the cerebrum was markedly above that of the pons-midbrain

section, in man this was not the case; a more limited rostral increase was found. In five studies on men, increases in carbonic anhydrase were found through the cord, medulla oblongata, and pons. These findings are charted in Fig. 2. The lower third of the medulla was included with the small piece of the cord obtained. The content for the upper two-thirds of the medulla oblongata was determined separately. The pons in three cases was divided into rostral and caudal sections which in turn were split into right and left parts.

A progressive increase was found from the upper cord to the rostral end of the pons. But in contrast to the animals studied, the content
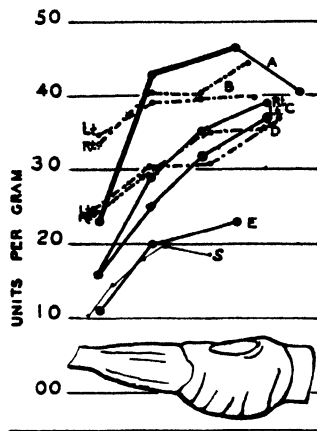
FIG. 2. In man, progression of carbonic anhydrase content through the cord plus one-third of the medulla, the upper two-thirds of the medulla, one area or two areas of the pons, and, in Case A, the brain stem. Progression through four consecutive areas of the medulla in a steer. Curve A, Case A, age 25 years; Curve B, Case 8179, age 42; Curve C, Case 8121, age 71; Curve D, Case 8150, age 82; Curve E, Case 8026, age 82; Curve S, steer. In Cases 8179, 8121, and 8150 separate determinations were made on material taken from the right and left sides of the specimens.

of the human cerebrum instead of being markedly higher was either lower or approximately equal to that of the rostral section of the pons. Samples from the frontal pole containing white and gray matter obtained by amputating a gyrus gave lower figures than did the pons; the similar mixed sample from the occipital pole of one brain also contained less enzyme, but the values for the occipital pole in the remaining four cases were higher than those for the pons.

Carbonic Anhydrase of Thalamus Compared with That of Cerebrum in Man—The relative position of the enzyme content of the thalamus was studied in five human brains. The content of the thalamus was compared with that of the subgyral white matter, the intragyral white matter, and

the cortex. The results are given in Table I with, in contrast, a few data from animals in all of which a good margin of difference in favor of the cerebrum is shown. In Case A values are given in detail from different regions of the hemisphere; the results from the remaining brains, for which in all twenty-three series of determinations were made, are averaged.

The content of the cortex was found to be lower than that of the thalamus, but the content of the intragyral white matter, the white matter immediately below the cortex, was in general higher.

TABLE I

Relationship of Carbonic Anhydrase Content of Thalamus to That of Pallium

Case No.	Thalamus	Carbonic anhydrase, units per gm				
		Pallium				
		Area	Subgyral	Intragyral	Cortex
A. Man	38.2	Frontal pole	37.1	42.0	25 5
		Superior frontal gyrus	37.6	43.9	32.8
		Parietal, area 7	39.3	43.5	35.3
		" " 19		54 1	40.7
		Occipital " 18	28.6	37 3	31.7
		" " 17	44.1	48 2	38.3
		Superior temporal gyrus		38.5	28.6
		Inferior " "	40.0	41 6	28.7
		Middle " "	37.8	40 2	29.4
		Temporal pole	36 6	34.4	19.8
7991. Man	30.1	Average	26 4	33 8	27.3
7995. "	20.0	"		23 3	19.9
8026. "	21 5	"	23.6	31.1	23.8
8121. "	24.7*	"	29.9	33.6	25.8
	23.4†				
Steer	11.3	13.5			
Hog	17.2	24.1			
Cat	32.7	51.6			
"	25.1	43.3			

* Sample from right side of thalamus.
† Sample from left side of thalamus.

Data on Distribution of Carbonic Anhydrase in White Matter—The rostral progression of carbonic anhydrase content was studied in white matter alone and data from four cerebrums are given in Table II. Five different levels, as measured from the periphery, were selected, although not all were studied in each brain. The lowest consisted of white matter passing the basal ganglia, next were the callosum and adjacent fibers, third the centrum semiovale, fourth and fifth the subgyral and intragyral white matter.

In the four brains there was definite progression in carbonic anhydrase

content as the cortex was approached, with probably an acceleration as the cortex was neared. Four other brains studied, all from patients described as "absolutely inaccessible" and three of them aged 82, showed a less well defined gradient. In the four more normal brains the ratios for the five categories considered, beginning with the white matter at the level of the basal ganglia and ending with that of the white matter immedi-

TABLE II

Carbonic Anhydrase Content in Human Cerebrum Sampled at Varying Depths beneath Cortex

Case No	Age	Approximate depth	Distribution	Average
	yrs		*units per gm.*	*units per gm.*
A	8	Level of basal ganglia	31.9, 32.2, 35.5	33.3
		Callosum	35.8 callosum, 35.5 corona radiata	35.6
		Subgyral white matter	41.5 frontal pole, 49.7 area 7*	45.4
		Intragyral white matter	42.9 " " 49.8 " 7	46.4
B	25	Callosum	30.0	30.0
		Centrum semiovale	33.7	33.7
		Subgyral white matter	37 0 frontal pole, 37 6 superior frontal gyrus, 36.6 temporal pole, 37 8 area 21, 40.0 area 20, 39.3 area 7	38.6
		Intragyral white matter	42 1 frontal pole, 43 9 superior frontal gyrus, 34.4 temporal pole, 40.2 area 21, 41 6 area 20, 43.5 area 7	41.6
8076	35	Callosum	34 7 splenium, 33 6 periventricular	34.1
		Subgyral white matter	44.5 frontal pole, 29 0 postcentral gyrus, 50 9 area 7, 40.9 area 17	41.3
		Intragyral white matter	45 3 frontal pole, 42 9 postcentral gyrus, 45.3 area 7, 56 7 area 17	48.1
8121	71	Callosum	21.9 genu, 24.6 corpus, 23 3 splenium	23.2
		Centrum semiovale	29.8 frontal area, 24.6, 21.7, 26.1, 26.6 subrolandic	24.8
		Subgyral white matter	29.8 frontal pole, 30 0 area 7	29.9
		Intragyral white matter	30.2 " " 36.9 " 7	33.5

* Areas 7, 20, and 21 are approximately Brodmann's areas.

ately below the cortex (on the basis of 30 for the callosum), averaged 28.1:30.0:32.9:37.9:41.6.

Carbonic Anhydrase Level of Cerebellum in Relation to Cerebrum—The enzyme content of the cerebrum was compared with that of the cerebellum in eight species of animals. In man the content of the cerebellum was approximately equal to or less than that of the frontal pole. In the four

hogs studied the content of the cerebellum was far below that of the cerebrum. In two cats it was approximately equal in the two areas, while in the steer, the guinea pig, and the chicken the carbonic anhydrase content of the cerebellum was definitely greater than that of the cerebrum. In the steer and a calf a very low content of the cerebrum gave the cerebellar finding a preponderance of 150 to 200 per cent. Although no large numbers of animals have been examined in each group, the results in man,
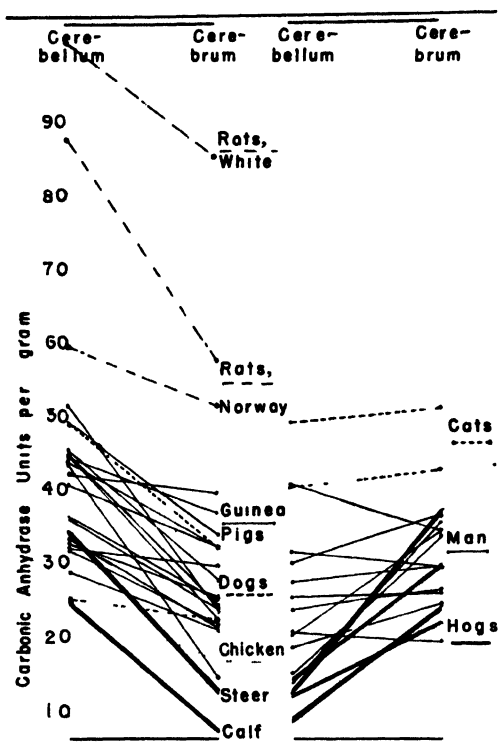
Fig 3   Relationship between the carbonic anhydrase content of the cerebrum and cerebellum in various species

guinea pigs, and hogs, which include the greatest number of analyses, support the suggestion that the relationship tends to vary with the species. These results are given in Fig. 3.

DISCUSSION

There tends to be considerable similarity in the pattern of quantitative distribution of carbonic anhydrase in the central nervous system in individuals of the same species, with differences among species. As an ex-

ample, the data on the two cats run parallel in five areas studied, while determinations in three areas in the central nervous system of a steer and a calf give parallel findings of a very different pattern from that seen in the cats. As has been previously reported, seven sections from two spinal cords of hogs gave no carbonic anhydrase not attributable to blood content ((1) Table III), while the cerebral content was fairly high and the cerebellum contained definitely less than the cerebrum. In guinea pigs, on the other hand, carbonic anhydrase was present in the cord, the content of the cerebrum was intermediate between that found in the hog and the low content of the steer, while the content of the cerebellum was greater than that of the cerebrum by approximately 50 per cent. The ratio of enzyme content of the cerebrum to that of the cerebellum tends to show a consistent species difference. In the guinea pig, the rat, and the steer, the cerebellar content is greater than that of the cerebrum; in the hog the reverse is the case. A consistent difference, to be demonstrated later, has also been found between the pattern of distribution of carbonic anhydrase in the cerebrum of man as compared with other animals studied.

A tendency for an increase in carbonic anhydrase in a rostral direction is illustrated in the spinal cords of the cat, the dog, and the guinea pig when more than one level of the cord has been examined. In the chicken, dog, cat, hog, and guinea pig the enzyme content of the cerebrum was definitely greater than that of the brain stem. But in man, as also in the steer, the cerebrum did not show a maximum enzyme content, although well defined gradients were found from the cord through the pons. The difference between the thalamus and the cerebrum was well defined in four animals studied in favor of the cerebrum. But in man, with five brains studied, the superiority of the cerebrum over the thalamus was generally limited to the white matter immediately below the cortex. The exception was found in the rolandic area, the enzyme content of which was below that of the thalamus. In man determinations on white matter alone show a progressive increase rostrally between white matter passing the basal ganglia to white matter immediately below the cortex.

Although exactly parallel data are not generally available, there seems to be a relationship between carbonic anhydrase content and oxygen uptake. This has been discussed elsewhere ((1) Table IV) in relation to findings by Dixon and Meyer and by Bass, in which the material studied was apparently the same as that upon which carbonic anhydrase determinations were made by me. There is also a parallelism between the relative oxygen uptake in the presence of succinate found in the brain of the cat, dog, rat, and man by Quastel and Wheatley (3) and the carbonic anhydrase content found by me in these animals, although the findings for the guinea pig are out of harmony. With the recent work by Craig and Beecher (4) on the O_2 uptake of the cortex, medulla, and cord of the

cat in which the ratios 100:34:12 were found, my results for carbonic anhydrase are in substantial agreement. The cortex and underlying white matter, which in the cat would reduce the ratio, were used by me, and instead of the medulla only, I used the medulla plus pons and midbrain. For these parts the ratios of carbonic anhydrase content were 100:68:39. My findings for the carbonic anhydrase content are in general in harmony with the extensive work of Himwich and Fazekas (5) on oxygen uptake and differential survival of levels of the central nervous system. It would seem that these parallelisms are more than a matter of chance and indicate that carbonic anhydrase plays an essential part in the metabolism of the central nervous system.

With respect to carbonic anhydrase in the blood, we have the situation in which carbon dioxide produced in all the tissues of the body has to be excreted in the comparatively small area of the lung. This is accomplished in the necessary time through the mediation of carbonic anhydrase (6). Although the total metabolism of the central nervous system is not of a different order from that of other active tissues and there is no condensation of the area through which excretion must take place, in nerve tissue there is a condensation in time not found in the needs of other tissues. The passage of a nerve impulse involves metabolism which must take place in milliseconds and it is possible that carbonic anhydrase may facilitate this extremely rapid metabolic progression. Mann, Tennenbaum, and Quastel (7) and more recently Nachmansohn and coworkers (8, 9) have related acetylcholine production to carbohydrate metabolism. Nachmansohn has postulated the thesis that the progress of a nerve impulse is due to the production and destruction of acetylcholine which produces changes in surface charge. If such a mechanism, dependent upon carbohydrate metabolism, is responsible for nerve conduction, it is conceivable that carbonic anhydrase may be a contributing factor to the rate of this progression.

Exactly how the enzyme might function cannot be postulated with our present incomplete knowledge of the events leading to the metabolism of carbohydrate in the brain. Two possibilities suggest themselves: one that the enzyme might facilitate the removal of CO_2, the other that it might produce a local pH, optimum for some enzyme functioning in the chain of reactions (suggested by Dr. Mary Maver). With reference to the removal of CO_2 I believe we lack evidence as to the state in which the CO_2 comes out of the metabolic chain, whether as CO_2 or in the ionized form. If it leaves as H_2CO_3, then conceivably carbonic anhydrase might be useful in changing the ionized form to CO_2, the form more easily escaping through the membrane. If it leaves as CO_2, then a local change to the ionized form might be useful to facilitate solution with a reversal at

the membrane. On the other hand if the enzyme were outside the neuron in accessory cells or in the sheath, it might facilitate the removal of CO_2 by changing the gas into the ionized form as it came through the cell membrane. The recent reversal of decarboxylation that has been demonstrated in other tissues with the labeled carbon atom (10) makes an inhibition by CO_2 seem a probability, while on the clinical side we have evidence of a similarity of effect of CO_2 and of the drugs which depress brain metabolism, as used in "narcoanalysis."

Carbonic anhydrase might contribute to the dominance of one level over another by making possible greater speed of stimulation at the dominant level or, on the other hand, for the stimulation of a larger number of nerve cells, an increase might be necessary upon subdivision of a fiber as the synapse is approached, to allow the production of energy adequate for the increased surface essential to this subdivision, since the conduction of a nerve impulse is a surface phenomenon.

SUMMARY

1 Carbonic anhydrase is found in the central nervous system of various species of animals in patterns which tend to be peculiar to the species.

2. Progressive rostral increases in carbonic anhydrase content have been found.

3. The enzyme is found in the white matter, in some instances in greater amount in the white matter than in the gray. It is possible that it functions more specifically with reference to the nerve fiber.

4. The findings are discussed in the light of their possible relationship to the functioning of the central nervous system.

BIBLIOGRAPHY

1 Ashby, W., *J Biol Chem*, **152**, 235 (1944)

2 Ashby, W, and Chan, D V, *J Biol Chem*, **151**, 515 (1943)

3 Quastel, J H, and Wheatley, A H M, *Biochem J*, **26**, 725 (1932)

4 Craig, F N, and Beecher, H K, *J Neurophysiol*, **6**, 135 (1943).

5. Himwich, II E, and Fazekas, J F, *Am J Physiol*, **132**, 454 (1941).

6. Roughton, F J W, *Physiol Rev*, **15**, 241 (1935)

7 Mann, P J F, Tennenbaum, M, and Quastel, J H, *Biochem J*, **27**, 1141 (1933).

8. Nachmansohn, D, Cox, R T, Coates, C W, and Machado, A. L, *J Neurophysiol*, **5**, 499 (1942)

9. Nachmansohn, D, and Machado, A L, *J Neurophysiol*, **6**, 397 (1943)

10 Evans, E A, Jr., *Science*, **96**, 25 (1942)

LETTERS TO THE EDITORS

REVERSIBLE INACTIVATION OF THE ADENOSINETRIPHOS-PHATASE ACTIVITY OF MYOSIN PREPARATIONS WITH COPPER AND CYANIDE

Sirs:

During the course of an investigation of the properties of human myosin isolated from patients with progressive muscular dystrophy it became necessary to devise methods which would assure constancy of the adenosine-triphosphatase activity of duplicate samples of a preparation. Traces of copper added to myosin preparations completely inhibited the enzymatic activity. The effect of copper was nullified by the addition of cyanide. Cyanide, however, increased the enzymatic activity of fresh preparations

TABLE I

Effect of Cyanide and Copper on Adenosinetriphosphatase Activity of Myosin Preparation

1 cc. of myosin solution containing 0.2 mg of N with adenosine triphosphate (270 γ of labile P) in 5 cc. of KCl-CO$_3$ buffer, 0 5 mg of CaCl$_2$ per cc. Incubated 10 minutes at 37°.

Preparation	P liber-ated	Preparation	P liber-ated
	γ		γ
Myosin	34	Myosin + copper sulfate	4
" + 0 04 M cysteine	48	" + " " + 0.005 M NaCN	41
" + 0 001 " NaCN	46	Oxidized myosin (foot-note 2)	
" + 0.005 " "	43	" " + 0.005 M NaCN	30
" + 0.01 " "	41	" " + 0.04 " cysteine	31
" + 0.1 " "	14		

of myosin (isolated by the procedure of Weber and Meyer[1]) as well as preparations which had been inactivated by oxidation with peroxide. The action of cyanide is quantitatively analogous to that of cysteine[2] except that higher concentrations of cyanide inhibit the enzymatic activity.

Preparations of myosin obtained by a procedure in which the myosin is extracted with a buffer containing copper and, after precipitation, re-

[1] Weber, H. H., and Meyer, K., *Biochem. Z.*, **266**, 137 (1933).
[2] Ziff, M., *J. Biol. Chem.*, **153**, 25 (1944).

dissolved in a buffer containing cyanide have been found to have a consistently higher enzymatic activity than those preparations in which cyanide buffer was used in the extraction. The preparations extracted with cyanide buffer, in turn, have a greater enzymatic activity than those prepared with the simple buffer, even though similar amounts of cyanide are present in the test of activity.

The data given in Tables I and II were obtained with preparations from rat muscle. The buffer containing copper was prepared by adding enough copper sulfate to saturate the buffer and the excess cupric hydroxide was removed by filtration.

TABLE II

Comparison of Methods of Extraction of Adenosinetriphosphatase Activity

1 cc. of myosin containing 0.2 mg. of N tested as in Table I.

Method of extraction	Activator added	P liberated
		γ
Plain buffer	0.001 M NaCN	45
Cyanide "	0.001 " "	71
Copper "	0.001 " "	82
" "	0.01 " "	133
" " .	0.03 " "	60

The data obtained are interpreted as being in agreement with the hypothesis that sulfhydryl groupings of the myosin molecule are functional units in the cleavage of phosphate from adenosine triphosphate. Detailed studies of the effects of copper and cyanide on the birefringence of flow of myosin and the utilization of copper in the purification of myosin will be presented in a future communication.

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EXTENSION OF THE THUNBERG TECHNIQUE FOR MEASURE-MENT OF DEHYDROGENASE ACTIVITY

Sirs:

Dehydrogenase activity is ordinarily measured in the presence of excess substrate by the rate of oxygen uptake in the presence of methylene blue (Warburg technique) or by the decolorization time of methylene blue in the absence of oxygen (Thunberg technique). Water-soluble dehydrogenases suffer surface denaturation and are inactivated during the rapid shaking required to maintain equilibrium with the vapor phase in the otherwise satisfactory Warburg technique. Accuracy in the Thunberg technique requires the addition of a constant amount of methylene blue and complete removal of oxygen from the reaction chamber. These drawbacks may be eliminated by the following device.

A 14 × 125 mm. Pyrex test-tube suitable for use in the Klett-Summerson photoelectric colorimeter is equipped with a 14/35 outer standard taper joint. A side arm with a glass stop-cock is sealed onto the tube in such a way that it will not interfere with use in the colorimeter. The arm of an inner 14/35 standard taper joint is sealed off and curved to 90° or more for a side bulb.

1 ml. of 5 per cent substrate is placed in the side bulb and heated to boiling for a few seconds. 5 ml. of 0.002 per cent methylene blue (or sufficient to give a reading of about 500) are similarly boiled in the main tube. Both are cooled to 37°. 0.5 ml. of tissue extract or enzyme preparation and 0.5 ml. of *"Kochsaft"* or codehydrogenase preparation are then added to the main tube. The side bulb is put in place and the tube evacuated to a pressure of 40 to 50 mm. of Hg. It is neither necessary nor desirable to remove all of the oxygen. The stop-cock is then closed and the contents of the tube mixed. It is then placed in the colorimeter, equipped with a No. 66 red filter. After 5 to 15 minutes the oxygen remaining in solution becomes exhausted and the methylene blue color decreases linearly with time except for small periods at the beginning and at the end of the curve. The rate of decrease of the colorimeter reading is a measure of the amount of dehydrogenase present. The apparatus may be calibrated with known amounts of methylene blue dissolved in 7 ml. of water, in terms of colorimeter units per mole of methylene blue per liter. Presence of red or brown color or finely suspended material in the enzyme preparation does not interfere with the method.

This apparatus has been successfully used to measure glutamic, glucose, succinic, citric, and alcohol dehydrogenases in various liver preparations.

Studies of the effect of substrate concentration may be made with this

apparatus by evacuating most of the oxygen and extrapolating the decolorization curve back to the time of mixing.

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A PHOSPHORYLATED DERIVATIVE OF PYRIDOXAL AS THE COENZYME OF TYROSINE DECARBOXYLASE

Sirs:

Pyridoxine derivatives have been shown to be involved in the decarboxylation of tyrosine.[1] More recently it has been demonstrated[2] that cell suspensions of *Streptococcus faecalis* R[3] harvested from a medium deficient in "pseudopyridoxine" are unable to decarboxylate tyrosine; yet they acquire this function upon the addition of pyridoxal.[4]

We have now been able to obtain a dried cell enzyme preparation from *Streptococcus faecalis* R grown in a medium deficient in "pseudopyridoxine" similar to that used previously.[5] This preparation decarboxylates tyrosine

Tyrosine Decarboxylation by Enzyme Preparations from Streptococcus faecalis R

Per Warburg cup, M/30 phthalate, M/180 tyrosine, 10 mg. *in vacuo* of dried cells; with Preparation III also M/2 phosphate; pH 5.0; 28°.

Additions per cup	Preparation I	Preparation II	Preparation III
	Q_{CO_2}	Q_{CO_2}	Q_{CO_2}
None	4.0	6.4 '	8.4
3 γ pyridoxal	4.5*	11.1	9.8
1 mg. ATP	4.0	4.1	6.0
Both .	20.5	39.7	57.2

* In quantities of pyridoxal up to 100 γ no increased stimulation was found.

very slowly, is slightly stimulated by the addition of pyridoxal, but is markedly stimulated if supplied with pyridoxal and adenosine triphosphate (ATP). As ATP supplied without pyridoxal has little effect (or is slightly inhibitory), these results would indicate that pyridoxal is converted into a phosphorylated derivative which is active as the coenzyme of tyrosine decarboxylase. The addition of muscle adenylic acid plus pyridoxal does not increase the rate above that with pyridoxal alone. The stimulation of tyrosine decarboxylation upon the addition of pyridoxal to living cells is attributed to the availability of ATP which may be used for its phosphorylation.

Treatment of pyridoxal with thionyl chloride and the removal of the

[1] Gunsalus, I. C., and Bellamy, W. D., *J. Bact.*, **47**, 413 (1944).

[2] Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944).

[3] American Type Culture Collection No. 8043, under name of *Streptococcus lactis* R.

[4] We wish to thank Dr. E. E. Snell, and Merck and Company, Inc., for samples of pyridoxal.

[5] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, in press (1944).

substituted chlorine with silver dihydrogen phosphate yield a material which possesses coenzyme activity for tyrosine decarboxylase in the absence of ATP. Similarly, an active compound can be synthesized by treatment of pyridoxal with phosphoric acid in the cold. These data indicate that the alcoholic hydroxyl of pyridoxal (position 5) is the group phosphorylated but the exact structure of the coenzyme remains to be determined.

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STUDIES ON METHODS OF INCREASING FOLIC ACID ACTIVITY IN LIVER FRACTIONS AND IN YEAST*

Sirs:

While studying the properties of vitamins B_{10} and B_{11} and folic acid,[1] we noted an unexplained increase in folic acid activity, as measured with *Streptococcus lactis* and *Lactobacillus casei*.[2] For example, treatment of various purified liver fractions in acid solution with ultraviolet light[3] often resulted in an actual increase in folic acid activity. Further work showed ᵗʰᵃt treatment of these fractions with acid or base alone caused a marked ⸱ ⸱ in their apparent folic acid content.

Material tested	Treatment*	Time	No. of samples	Folic acid activity†
		hrs.		γ *per gm.*
Preparation 214H	Control		4	0.07 (0.05–0.09)
	pH 3	4	3	0.69 (0.63–0.75)
	" 3 (boiling)	4	1	0.45
	" 4	4	2	0.71 (0.61–0.80)
	" 4	6	1	0.85
	" 7	4	1	0.10
	0.1 N KOH	4	1	0.10
	2 N KOH	1	1	0.81
Yeast (Anheuser-Busch, Strain C)	Control		3	0.28 (0.18–0.34)
	pH 3	4	2	6.8 (5.2 –8.3)
	2 N KOH	1	2	4.6 (3.6 –6.0)
	Taka-diastase (37°), pH 4.5	24	1	5.8
	pH 4.5 (37°)	24	1	5.4

* All samples were autoclaved at 15 pounds pressure unless otherwise noted.
† Expressed in terms of the standard, vitamin B_c, per gm. of starting material (or its equivalent).

The best results which we have obtained with a typical fraction from liver (Preparation 214H, a dialysis residue of the 75 per cent acid alcohol filtrate of the Super Filtrol eluate[1]) and from yeast are given in the table.

[1] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **153**, 423 (1944).
[2] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **152**, 157 (1944).
[3] Mitchell, H. K., and Williams, R. J., *J. Am. Chem. Soc.*, **66**, 271 (1944).

Autoclaving the sample with either acid (pH 3 or 4) or 2 N KOH caused the optimal increase in *S. lactis* activity. In each case the *L. casei* activity corresponded well with the *S. lactis* value; however, there was even a greater increase with yeast as measured with *L. casei* (a 42-fold increase at pH 3, 4 hours). Other substances, such as Liver Fraction L and certain purified liver fractions, also gave appreciable increases. The presence of a reducing agent such as ascorbic acid or $Na_2S_2O_4$ during autoclaving under the acid conditions appeared to favor the increase.

The Texas group[4] has shown that the apparent folic acid content of various materials may be increased several fold by treatment with takadiastase at pH 4.5 to 4.7. We have tested our Preparation 214H and yeast under these conditions and found no increase in *S. lactis* or *L. casei* activity which could not be accounted for by treatment with acid alone (see the table). Likewise Binkley *et al.*[5] have reported (as the Texas group[3] and we[2] have indicated) that certain yeast and liver extracts contained a "conjugate" of vitamin B_c which had high microbiological activity following "enzymatic digestion." Again, as is seen in the table, it is entirely possible that the increase in activity was due to the acid or base present and not to the enzyme itself.

It is impossible to tell at the present time whether the increase is due entirely to an actual liberation of bound folic acid or to a modification of the molecule. Further work on this problem and on the relation of the increase in microbiological activity to vitamins B_{10} and B_{11} will appear in a later paper.

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[4] Cheldelin, V. H., Eppright, M. A., Snell, E. E , and Guirard, B. M., *Univ. Texas Pub., No. 4237*, 15 (1942)

[5] Binkley, S. B , Bird, O. D., Bloom, E. S., Brown, R A , Calkins, D. G., Campbell, C. J., Emmett, A D., and Pfiffner, J. J., *Science*, **100**, 36 (1944).

ISOLATION OF A PEPTIDE OF p-AMINOBENZOIC ACID FROM YEAST

Sirs:

p-Aminobenzoic acid (PAB) specifically reverses the bacteriostatic action of the sulfonamides.[1] The view is currently held that the bacteriostatic effect is the result of competitive inhibition by sulfonamides of an enzymatic process in which PAB participates. We undertook an investigation of the forms in which PAB occurs in yeast in the hope that the information so gained might provide a clue to possible enzymatic functions of PAB.

We have isolated from 50 kilos of dried yeast[2] about 400 mg. of a polypeptide (over-all yield 2 per cent) which contains 8.0 per cent PAB and 10.5 per cent N. The main steps in the isolation procedure are (1) precipitation of the peptide as the silver salt from the neutralized trichloroacetic acid filtrate of dried yeast suspensions, (2) precipitation as the lead salt from an acidified aqueous alcohol solution of the silver salt, (3) conversion of the lead salt to the barium salt, and finally (4) fractional precipitation of the free peptide from alcohol-ether or alcohol-acetone mixtures.

Blanchard, who was the first to isolate free PAB from yeast,[3] has presented evidence to indicate that "yeast also contains a combined form of p-aminobenzoic acid from which this substance is liberated during autolysis." It appears very likely that the peptide which we have isolated is identical with his combined form of PAB. The peptide has no antisulfonamide activity but free PAB is liberated by hydrolysis in strong acid or alkali and then shows the characteristic antisulfonamide effect. In order to confirm the identity of the PAB component, a sample of the peptide containing 10.4 mg. of bound PAB was hydrolyzed in 20 per cent HCl at 100°. Liberated PAB in 70 per cent yield was separated by ether extraction and converted to the picryl derivative in 74 per cent yield. M.p. (after three recrystallizations) 281–283°, uncorrected; m.p. of authentic p-picrylaminobenzoic acid 284–285°, uncorrected; mixed m.p. 281–283°, uncorrected. Analysis of a 2.7 mg. sample showed C 44.0 per cent, H 2.95; theory, C 44.8 per cent, H 2.3.

l-Glutamic acid hydrochloride was isolated in 50 per cent yield from 214 mg. of a less pure preparation. $[\alpha]_D^{27} = +25.4°$ (2.4 per cent solution in 1 N HCl). Nitrogen (Kjeldahl) 7.7 per cent; theory 7.6. At least 80

[1] Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).

[2] We are indebted to Dr. Charles N. Frey of The Fleischmann Laboratories for a very generous supply of dried yeast.

[3] Blanchard, K. C., *J. Biol. Chem.*, **140**, 919 (1941).

per cent of the non-PAB nitrogen has been accounted for as glutamic acid by the specific method of Cohen.[4]

The polypeptide is strongly acidic, containing approximately one carboxyl group for each nitrogen atom. It dialyzes readily without change in analytical characteristics and is very soluble both in water and ethyl alcohol. On the basis of the evidence already available, the structure of the compound can be formulated as a chain of 10 to 12 glutamic acid residues, which is linked to the carboxyl group of 1 PAB molecule. Since PAB in the polypeptide can be estimated by the Bratton and Marshall diazotization procedure[5] without preliminary hydrolysis, the amino group must be presumed to be free.

Indirect evidence for the existence in liver of a similar bound form of PAB has been obtained but the extremely low concentration of this bound form did not encourage us to undertake its isolation.

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[4] Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
[5] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).

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